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(54) METHOD FOR EVALUATING EFFECT ON IMPROVING INSULIN RESISTANCE

**(57)Abstract:**

**PROBLEM TO BE SOLVED:** To treat and improve insulin resistance by finding the target gene of an insulin resistance improver and using the gene and its product.

**SOLUTION:** Effect on improving insulin resistance of a test sample is evaluated by using adipsin or an expressed amount of adipsin gene as an index in the test sample under an administration condition.

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**CLAIMS**

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[Claim(s)]

[Claim 1]

How to evaluate the insulin resistance improvement effect of this examined substance by making ADIPUSHIN or the amount of ADIPUSHIN gene expression in the specimen under the administration conditions of an examined substance into an index.

[Claim 2]

How to evaluate the insulin resistance improvement effect by this examined substance by measuring ADIPUSHIN or the amount of ADIPUSHIN gene expression in administration of an examined substance, and the specimen under the conditions of not prescribing a medicine for the patient.

[Claim 3]

The assessment approach of the insulin resistance improvement effect including the following process of an examined substance :

- 1) Breed an animal under administration of an examined substance, or the conditions of not prescribing a medicine for the patient.;
- 2) Detect the amount of ADIPUSHIN gene expression in the blood of the above-mentioned animal, or a cell.;
- 3) Evaluate the insulin resistance improvement effect of this examined substance based on administration of an examined substance, or a difference of the amount of ADIPUSHIN gene expression under the conditions of not prescribing a medicine for the patient.

[Claim 4]

The approach according to claim 3 of including further the process which extracts all RNA from the inside of blood or a cell in said approach.

[Claim 5]

The approach according to claim 3 or 4 characterized by detecting the amount of gene expression by any one approach chosen from a gene chip, a cDNA array and the nucleic acid hybridization method using the solid

phase-ized sample chosen from a membrane filter, RT-PCR method, the real-time PCR method, a subtraction technique, the differential displaying method, a differential hybridization method, and a cross hybridization method.

[Claim 6]

The approach according to claim 3 or 4 that the amount of gene expression is characterized by being detected by RT-PCR method and the real-time PCR method.

[Claim 7]

The assessment approach of the insulin resistance improvement effect including the following process of an examined substance :

- 1) Breed an animal under administration of an examined substance, or the conditions of not prescribing a medicine for the patient.;
- 2) Detect the blood of the above-mentioned animal, or the amount of manifestations of ADIPUSHIN in a cell using the antibody specifically combined with this ADIPUSHIN.;
- 3) Evaluate the insulin resistance improvement effect of this examined substance based on a difference of administration of an examined substance or the amount of manifestations of ADIPUSHIN under the conditions of not prescribing a medicine for the patient.

[Claim 8]

The approach according to claim 7 characterized by detecting the amount of manifestations of ADIPUSHIN by any one approach chosen from a Western blot technique, dot blotting methods, slot blotting methods, the ELISA method, and the RIA method.

[Claim 9]

The approach according to claim 7 that the amount of manifestations of ADIPUSHIN is characterized by being detected by the Western blot technique.

[Claim 10]

An approach given in any 1 term of claims 3-9 whose cell is a liver cell.

[Claim 11]

An approach given in any 1 term of claims 3-10 whose animal is a type 2 diabetes model animal.

[Claim 12]

The approach according to claim 11 an animal is a mouse.

[Claim 13]

The assessment approach of the insulin resistance improvement effect including the following process of an examined substance :

- 1) Cultivate a cell under administration of a specimen material, or the conditions of not prescribing a medicine for the patient.;
- 2) Detect the amount of ADIPUSHIN gene expression in the

above-mentioned cell, or detect the amount of manifestations of ADIPUSHIN using the antibody specifically combined with this ADIPUSHIN.;

3) Evaluate the insulin resistance improvement effect of this examined substance based on a difference of administration of a specimen material, the ADIPUSHIN gene under the conditions of not prescribing a medicine for the patient, or the amount of manifestations of ADIPUSHIN.

[Claim 14]

The kit for assessment of the insulin resistance improvement effect containing at least one or more of an examined substance chosen from the group which consists of following a-e.

a) The oligonucleotide primer which 15 – 30 base length for amplifying specifically an ADIPUSHIN gene (the array number 1 or array number 12) followed

b) The polynucleotide probe with which the 20 – 1500 base length for combining with an ADIPUSHIN gene specifically and detecting this gene continued

c) The solid phase-ized sample to which the polynucleotide probe given [ above-mentioned ] in b was fixed

d) The antibody for combining with ADIPUSHIN (the array number 2 or array number 13) specifically, and detecting this ADIPUSHIN

e) The second antibody which can be specifically combined with an antibody given [ above-mentioned ] in d

[Claim 15]

The insulin resistance improvement agent containing Homo sapiens ADIPUSHIN or a Homo sapiens ADIPUSHIN gene.

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[Translation done.]

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**DETAILED DESCRIPTION**

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[Detailed Description of the Invention]

[Field of the Invention]

[0001]

This invention relates to the kit for assessment and insulin resistance improvement agent of the assessment approach of the insulin resistance improvement effect of an examined substance of having used ADIPUSHIN or an ADIPUSHIN gene, and the insulin resistance improvement effect of this examined substance.

[Background of the Invention]

[0002]

The number of patients is increasing in recent years, and diabetes mellitus attracts attention as one of the adult diseases. Although there are insulin-dependent type 1 diabetes and type 2 diabetes of non-dependence in diabetes mellitus, type 2 diabetes (insulin non-dependency diabetes mellitus, NIDDM) is the type of many diabetes mellitus to Japanese people, and early detection and early treatment are important for it from the point of the prognosis.

[0003]

However, since the origin of type 2 diabetes is various, the knowledge about the cause expected is scarce. As a cause of the lack of an insulin operation in type 2 diabetes, the abnormalities of an insulin susceptibility device and lowering of insulin secretion are cited. In Europe and America, although the former, i.e., insulin resistance, is the main causes of type 2 diabetes, there are not little many in Japan, also when insulin hyposecretion is the main causes.

[0004]

As oral diabetic medicine, although the insulin secretion accelerator of a sulfonyl urea system is used for many years, the insulin resistance improvement agent is also developed in recent years. "Insulin resistance

improvement agents" is drugs which compensate the lack of an operation of an insulin and improve insulin resistance by reinforcing the susceptibility of an insulin receptor. As such an insulin resistance improvement agent For example, troglitazone (for example, patent reference 1 reference), pioglitazone (For example, the patent reference 2 – 4 reference), ROSHIGURITAZON (for example, five to patent reference 7 reference), GI-262570 (for example, patent reference 8 reference), JTT-501 (Patent reference 9 – 11 reference [ for example, ]) AZ-242 (for example, 12 to patent reference 14 reference), MCC-555 (for example, 15 to patent reference 17 reference), YM-440 (Patent reference 18 – 20 reference [ for example, ]) KRP-297 (for example, the patent reference 21 and 22 reference), T-174 (for example, 23 to patent reference 25 reference), NC-2100 (Patent reference 26 – 28 reference [ for example, ]) NN-622 (for example, the patent reference 29 and 30 reference), The salt permitted on BMS-298585 (for example, patent reference 31 reference) and 5-[4-(6-methoxy-1-methylbenzimidazol-2-ylmethoxy) benzyl] thiazolidine -2, 4-dione, and its pharmacology (For example, 32 to patent reference 34 reference) etc. — an oxazole compound, an OKISA diazo lysine compound, a thiazolidine compound, or a phenoxazine compound can be mentioned.

[0005]

Thiazolidine derivative like ROJIGURITAZON and pioglitazone among these insulin resistance improvement agents is already used by the clinical field (for example, nonpatent literature 1-3 and patent reference 35 reference). These drugs are characterized by having a thiazolidine ring frame as basic structure, and combine PPARgamma which is a receptor in a nucleus as a target molecule. And it is thought by changing the gene expression in liver, muscles, and a fat cell that a type 2 diabetes patient's insulin resistance is improved.

[0006]

In the fat tissue which are the main manifestation organs of PPARgamma, the direct action of the insulin resistance improvement agent is carried out to PPARgamma, and it rises the insulin susceptibility of fat tissue (for example, nonpatent literature 4 reference). On the other hand, the insulin resistance improvement agent which uses PPARgamma as a target molecule promotes sugar utilization in liver, and controls sugar bleedoff with muscles. However, there are many still unknown points about whether an improvement of the insulin resistance in muscles or liver is the direct operation over PPARgamma discovered to muscles or liver (for example, nonpatent literature 5 reference).

[0007]

Recently, it adjusts a living body's insulin susceptibility that the insulin resistance improvement agent represented by thiazolidine derivative adjusts

the amount of production of the factor secreted from fat tissue, and it is considered to be the cause which improves type 2 diabetes. Fat tissue is not the storage organ of a mere fat but an endocrine organ which secretes many factors. In these secretor factor leptin which adjusts abstemious diet and an energy metabolism (leptin) (nonpatent literature 6 and 7 reference) The tumor necrosis factor to which insulin susceptibility is reduced ( ) [ tumor necrosis factor:TNF-alpha ] (For example, nonpatent literature 8 reference) and REJISUCHIN ( ) [ resistin] (For example, nonpatent literature 9 reference); the factor of a large number relevant to obesity, such as ADIPONE cutin (adiponectin (for example, nonpatent literature 10 and 11 reference)) which accelerates insulin susceptibility, insulin resistance, and type 2 diabetes is contained.

[0008]

For example, the manifestation is going up in the fat tissue of the mouse with which TNF-alpha presents obesity and diabetes mellitus, and manifestation sthenia of TNF-alpha accepted with obesity and a diabetes-mellitus model mouse is controlled by neutralizing TNF-alpha in the condition which insulin resistance improves that insulin resistance has improved by pioglitazone administration again (for example, nonpatent literature 12 reference) (for example, nonpatent literature 13 reference).

[0009]

Conversely, in the mouse and Homo sapiens whom ADIPONE cutin presents obesity and diabetes mellitus, the manifestation is falling (for example, nonpatent literature 14 and 15 reference), and the manifestation of ADIPONE cutin goes up at the time of insulin resistance improvement agent administration (for example, nonpatent literature 16 and 17 reference).

[0010]

ADIPUSHIN is protein produced by the large quantity by the fat cell like ADIPONE cutin (for example, nonpatent literature 18 reference). However, clinical meaning of ADIPUSHIN is not clarified yet and relation with diabetes mellitus or insulin resistance is not reported, either.

[Patent reference 1] JP,60-051189,A official report

[Patent reference 2] JP,61-267580,A

[Patent reference 3] The Europe patent No. 193,256 description

[Patent reference 4] U.S. Pat. No. 4,687,777 description

[Patent reference 5] Patent Publication Heisei No. 512249 [ nine to ] official report

[Patent reference 6] International disclosure/[ 95th ] No. 21608 pamphlet

[Patent reference 7] U.S. Pat. No. 5,002,953 description

[Patent reference 8] International disclosure/[ 00th ] No. 8002 pamphlet

[Patent reference 9] International disclosure/[ 95th ] No. 18125 pamphlet

[Patent reference 10] The Europe patent application disclosure No. 684,242



description

[0011]

[Patent reference 11] U.S. Pat. No. 5,728,720 description

[Patent reference 12] International disclosure/[ 99th ] No. 62872 pamphlet

[Patent reference 13] The Europe patent application disclosure No. 1,084,103  
description

[Patent reference 14] U.S. Pat. No. 6,258,850 description

[Patent reference 15] JP,6-247945,A

[Patent reference 16] The Europe patent application disclosure No. 604,983  
description

[Patent reference 17] U.S. Pat. No. 5,594,016 description

[Patent reference 18] International disclosure/[ 94th ] No. 25448 pamphlet

[Patent reference 19] The Europe patent No. 696,585 description

[Patent reference 20] U.S. Pat. No. 5,643,931 description

[0012]

[Patent reference 21] JP,10-87641,A

[Patent reference 22] U.S. Pat. No. 5,948,803 description

[Patent reference 23] JP,64-56675,A

[Patent reference 24] The Europe patent No. 283,035 description

[Patent reference 25] U.S. Pat. No. 4,897,393 description

[Patent reference 26] JP,9-100280,A

[Patent reference 27] The Europe patent application disclosure No. 787,725  
description

[Patent reference 28] U.S. Pat. No. 5,693,651 description

[Patent reference 29] International disclosure/[ 99th ] No. 19313 pamphlet

[Patent reference 30] U.S. Pat. No. 6,054,453 description

[0013]

[Patent reference 31] International disclosure/[ 01st ] No. 21602 pamphlet

[Patent reference 32] The Europe patent application disclosure No. 745,600  
description

[Patent reference 33] JP,9-295970,A

[Patent reference 34] U.S. Pat. No. 5,886,014 description

[Patent reference 35] JP,60-051189,A

[0014]

[Nonpatent literature 1] "Life science (Life Science) 2000;67:p2405-2416"

[Nonpatent literature 2] "A Japanese clinical" 2000;58:p389-404

[Nonpatent literature 3] "Pharma KOSERAPI (Pharmacotherapy) 2001;  
21:p.1082-1099"

[Nonpatent literature 4] "And KURAI slag G (Endocrinology) 1996;  
137:p.1984-1990"

[Nonpatent literature 5] "And KURAI slag G (Endocrinology) 1998;  
139:p.5034-5041"

[Nonpatent literature 6] "Nature (Nature) 1994; 372:p.425-432"

[Nonpatent literature 7] "Nature (Nature) 1998; 395:p.763-770"

[Nonpatent literature 8] "Nature (Nature) 1997; 389:p.610-614"

[Nonpatent literature 9] "Nature (Nature) 2001; 409:p.307-312"

[Nonpatent literature 10] "Nature Mehdi Soon (Nature Medicine) 2001; 7:p.941-946"

[0015]

[Nonpatent literature 11] "Nature Mehdi Soon (Nature Medicine) 2001; 7:p.947-953"

[Nonpatent literature 12] "Science (Science) 1993; 259:p.87-91"

[Nonpatent literature 13] "And KURAI slag G (Endocrinology) 1994; 134:p.264-270"

[Nonpatent literature 14] "Journal of Biological Chemistry (Journal of Biological Chemistry) 1996; 271:p.10697-10703"

[Nonpatent literature 15] "Biochemistry - and - biotechnology physics research communication (Biochemistry and Biophysics Research Communication) 1999; 257:p.79-83"

[Nonpatent literature 16] "Nature Mehdi Soon (Nature Medicine) 2001; 7:p.941-946"

[Nonpatent literature 17] "Nature Mehdi Soon (Nature Medicine) 2001; 7:p.947-953"

[Nonpatent literature 18] "NUKUREIKKU acid research (Nucleic Acids Research)" 1986;25;14 (22):p.8879-8892

[Description of the Invention]

[Problem(s) to be Solved by the Invention]

[0016]

This invention aims at offering the kit for assessment and insulin resistance improvement agent of the assessment approach of the insulin resistance improvement effect of an examined substance, and the insulin resistance improvement effect of this examined substance by using a header, this gene, and its product for the target gene of an insulin resistance improvement agent.

[Means for Solving the Problem]

[0017]

In order that this invention persons may solve the above-mentioned technical problem, as a result of inquiring wholeheartedly, it is the diabetes-mellitus model mouse which prescribed the insulin resistance improvement agent for the patient, and found out that the manifestation of ADIPUSHIN was guided notably. Furthermore, when the superfluous manifestation of ADIPUSHIN was carried out by the liver of the mouse concerned, the blood sugar level and the insulin value in blood fell, and it found out that the indication of an insulin resistance improvement appeared.

From these results, this invention persons came to complete a header and this invention for ADIPUSHIN serving as insulin resistance and an index of the improvement.

[0018]

Namely, this invention offers the approach of evaluating the insulin resistance improvement effect of this examined substance, by making ADIPUSHIN or the amount of ADIPUSHIN gene expression in the specimen under the administration conditions of an examined substance into an index. It may set to said approach and assessment may be performed by [ with ADIPUSHIN in administration of an examined substance, and the specimen under the conditions of not prescribing a medicine for the patient, or the amount of ADIPUSHIN gene expression ] comparing.

[0019]

In one embodiment [I], the approach of this invention includes the following process.

- 1) Breed an animal under administration of an examined substance, or the conditions of not prescribing a medicine for the patient.;
- 2) Detect the amount of ADIPUSHIN gene expression in the blood of the above-mentioned animal, or a cell.;
- 3) Evaluate the insulin resistance improvement effect of this examined substance based on administration of an examined substance, or a difference of the amount of ADIPUSHIN gene expression under the conditions of not prescribing a medicine for the patient.

[0020]

Here, said process 2 may detect the amount of ADIPUSHIN gene expression from all these RNA further including the process which extracts all RNA from the inside of blood or a cell.

[0021]

Said amount of ADIPUSHIN gene expression can be detected by any one approach chosen from a gene chip, a cDNA array and the nucleic acid hybridization method using the solid phase-ized sample chosen from a membrane filter, RT-PCR method, the real-time PCR method, a subtraction technique, the differential displaying method, a differential hybridization method, and a cross hybridization method, and RT-PCR method and its real-time PCR method are especially desirable.

[0022]

Moreover, in another embodiment [II], the approach of this invention includes the following process.

- 1) Breed an animal under administration of an examined substance, or the conditions of not prescribing a medicine for the patient.
- 2) Detect the blood of the above-mentioned animal, or the amount of manifestations of ADIPUSHIN in a cell using the antibody specifically

combined with this ADIPUSHIN.;

3) Evaluate the insulin resistance improvement effect of this examined substance based on a difference of administration of an examined substance or the amount of manifestations of ADIPUSHIN under the conditions of not prescribing a medicine for the patient.

[0023]

Here, the amount of manifestations of said ADIPUSHIN can be detected by any one approach chosen from a Western blot technique, dot blotting methods, slot blotting methods, the ELISA method, and the RIA method, and especially its Western blot technique is desirable.

[0024]

As for a cell, in said mode [I] and mode [II], it is desirable to use a liver cell. Moreover, as for an animal, it is desirable to use a type 2 diabetes model animal, especially a type 2 diabetes model mouse.

[0025]

Furthermore, in another embodiment [III], the approach of this invention includes the following process.

- 1) Cultivate a cell under administration of a specimen material, or the conditions of not prescribing a medicine for the patient.;
- 2) Detect the amount of ADIPUSHIN gene expression in the above-mentioned cell, or detect the amount of manifestations of ADIPUSHIN using the antibody specifically combined with this ADIPUSHIN.;
- 3) Evaluate the insulin resistance improvement effect of this examined substance based on a difference of administration of a specimen material, the ADIPUSHIN gene under the conditions of not prescribing a medicine for the patient, or the amount of manifestations of ADIPUSHIN.

[0026]

This invention offers the kit for assessment of the insulin resistance improvement effect of an examined substance. This kit contains at least one or more chosen from the group which consists of following a-e,

- a) The oligonucleotide primer which 15 – 30 base length for amplifying specifically an ADIPUSHIN gene (the array number 1 or array number 12) followed
- b) The polynucleotide probe with which the 20 – 1500 base length for combining with an ADIPUSHIN gene specifically and detecting this gene continued
- c) The solid phase-ized sample to which the polynucleotide probe given [ above-mentioned ] in b was fixed
- d) The antibody for combining with ADIPUSHIN (the array number 2 or array number 13) specifically, and detecting this ADIPUSHIN
- e) The second antibody which can be specifically combined with an antibody given [ above-mentioned ] in d

Furthermore, this invention offers the insulin resistance improvement agent containing Homo sapiens ADIPUSHIN or a Homo sapiens ADIPUSHIN gene.

[Effect of the Invention]

[0027]

It was shown that it can become a new index for ADIPUSHIN to evaluate insulin resistance and its improvement by this invention. Simple screening of an index, then an insulin resistance improvement agent can be performed for this ADIPUSHIN or ADIPUSHIN gene expression. Moreover, insulin resistance can also be treated by medicating with this ADIPUSHIN or an ADIPUSHIN gene the patient who has insulin resistance.

[Best Mode of Carrying Out the Invention]

[0028]

This invention relates to the assessment approach of the insulin resistance improvement effect of an examined substance and insulin resistance improvement agent which made the index ADIPUSHIN or the amount of ADIPUSHIN gene expression in a specimen.

## 1. ADIPUSHIN

### 1.1 ADIPUSHIN and Insulin Resistance

It is reported that "ADIPUSHIN" concerning this invention is one of the secretion proteins mainly produced by the large quantity by the mammalian fat cell, and it is the homologue of the complement factor D (complement factor D) (White RT et al, J.Biol.Chem.1992, May 5, 267(13) 9210-9213).

[0029]

Although especially the origin of the ADIPUSHIN or the ADIPUSHIN gene which are used by this invention is not limited, the thing of the mammalian origin is desirable, the thing of the primates and the GETSU gear-tooth animal origin is more desirable, and Homo sapiens ADIPUSHIN and mouse ADIPUSHIN by which the function and amino acid sequence, and a base sequence are often studied especially are the most desirable.

[0030]

As shown in the example mentioned later, this invention persons checked that ADIPUSHIN gene expression moreover increased to a dosage dependence target notably in the type 2 diabetes model animal which prescribed the insulin resistance improvement agent for the patient. Furthermore, when the superfluous manifestation of ADIPUSHIN was carried out with the mouse concerned, it checked that an improvement of insulin resistance was found. These results show that ADIPUSHIN and its amount of gene expression can serve as an index by which insulin resistance and its improvement are evaluated.

[0031]

### 1.2 ADIPUSHIN Protein

ADIPUSHIN is secreted as mature protein, after imprinting as a precursor

containing transit peptide and separating this transit peptide part. Although the amino acid sequence (transit peptide \*\*\*\*) of Homo sapiens ADIPUSHIN is shown in the array number 2 among the ADIPUSHIN proteins concerning this invention, it is not limited to this array, but it is contained in Homo sapiens ADIPUSHIN of this invention as long as the peptide in which 1 or some amino acid are shown in deletion and the array permuted or added in this array also has a function as Homo sapiens ADIPUSHIN. Similarly, although the amino acid sequence of mouse ADIPUSHIN is shown in the array number 13, it is not limited to this array, but it is contained in mouse ADIPUSHIN of this invention as long as the peptide in which 1 or some amino acid are shown in deletion and the array permuted or added in this array also has a function as mouse ADIPUSHIN.

In addition, all of mature protein that have the precursor which has transit peptide, and transit peptide shall be included [ be / it / under / this / description / setting ] in the vocabulary "ADIPUSHIN."

[0032]

### 1.3 ADIPUSHIN Gene

Although the array of mRNA which carries out the code of Homo sapiens ADIPUSHIN among the ADIPUSHIN genes concerning this invention is shown in the array number 1, that gene is contained in the Homo sapiens ADIPUSHIN gene of this invention, as long as it is not limited to this array but the code of Homo sapiens ADIPUSHIN is carried out. Although similarly the array of mRNA which carries out the code of mouse ADIPUSHIN is shown in the array number 12, the gene is contained in the mouse ADIPUSHIN gene of this invention as long as the code of mouse ADIPUSHIN is carried out.

[0033]

In addition, not only DNA but the mRNA, cDNA, and cRNA shall be contained [ be / it / under / this / description / setting ] in the vocabulary a "gene." Therefore, DNA of ADIPUSHIN, mRNA(s), cDNA(s), and all the cRNA(s) are contained in the "ADIPUSHIN gene" concerning this invention.

[0034]

### 2 How to Evaluate Insulin Resistance Improvement Effect

This invention offers the approach of evaluating the insulin resistance improvement effect of this examined substance, by making ADIPUSHIN or the amount of ADIPUSHIN gene expression in the specimen under the administration conditions of an examined substance into an index.

[0035]

Said approach may measure and evaluate ADIPUSHIN or the amount of ADIPUSHIN gene expression in the specimen under the administration and the conditions of not prescribing a medicine for the patient, about one examined substance, and may be the same comparative evaluation about two

or more examined substances. Or as long as correlation of ADIPUSHIN or the amount of ADIPUSHIN gene expression, and an insulin resistance improvement effect is established experientially, based on the relation, the insulin resistance improvement effect of an examined substance may be evaluated without comparison contrast on an absolute scale.

[0036]

In the assessment approach of this invention, an insulin resistance improvement effect may evaluate the amount of manifestations of ADIPUSHIN as an index, and may evaluate the amount of ADIPUSHIN gene expression as an index. Moreover, an assessment system may be an in vivo system which used the animal, and may be an in vitro system using a cultured cell.

[0037]

In the approach of this invention, a "specimen" means the sample in which the ADIPUSHIN gene of this invention is contained for the blood isolated from a cultured cell, its extract, or an animal, body fluid, an organization, a cell, excrement, or those extracts. Especially as a specimen, the cell blood, or ADIPUSHIN and its gene are carrying out [ the cell ] the high manifestation is desirable, and a fat cell and a liver cell are the most desirable.

Moreover, administration for a living thing, the addition to culture medium, etc. shall contain all of making the condition that an examined substance exists in a specimen with "administration" of an examined substance.

[0038]

Each of the assessment approach which made the index hereafter the assessment approach which made the ADIPUSHIN gene the index, and ADIPUSHIN (protein) is explained concretely.

#### 2.1 Assessment Approach of Examined Substance Which Made ADIPUSHIN Gene Index (Inch Vivo System)

As for the assessment approach of the insulin resistance improvement effect of the examined substance in in vivo which made the ADIPUSHIN gene the index, it is desirable to include the following process.

Process 1: Breed an animal on condition that administration of an examined substance, or un-prescribing a medicine for the patient.

Process 2: Detect the amount of ADIPUSHIN gene expression in the blood of the above-mentioned animal, or a cell.

Process 3: Evaluate the insulin resistance improvement effect of this examined substance based on administration of an examined substance, or a difference of the amount of ADIPUSHIN gene expression under the conditions of not prescribing a medicine for the patient.

[0039]

Process 1: Breeding of an animal

Although especially the "animal" used by the approach of this invention is not limited, the type 2 diabetes model animal which presents insulin resistance is desirable. Such an animal may be a commercial thing or could be produced according to the well-known approach. As a commercial type 2 diabetes model animal, it is KK mice (for example, KK/Ta mouse, KK/San mouse, etc.), KK-Ay mice (for example, KK-Ay/Ta mouse etc.), C57 BL/KsJ-db/db mouse, C57 BL/KsJ-db/+m mouse, and C57 BL/KsJ-+m/+m, for example. Type 2 diabetes model rats, such as type 2 diabetes model mice, such as a mouse and an ob/ob mouse, and GK rat, etc. can be mentioned. These mice and rats can be purchased for example, from Japanese Clare, Inc.

[0040]

Said animal performs administration of an examined substance, or period breeding suitable under the conditions of not prescribing a medicine for the patient. Especially the dose of the examined substance to an animal is not limited, but should just set up a dosage suitably according to the description of an examined substance, or the weight of an animal. Moreover, what is necessary is not to limit especially the medication method and administration period of an examined substance to an animal, either, but just to set up the route of administration and administration period suitably according to the description of an examined substance.

[0041]

Process 2: Detection of an ADIPUSHIN gene

Next, blood or a cell is isolated from the animal bred under administration of an examined substance, or the conditions of not prescribing a medicine for the patient, and the amount of ADIPUSHIN gene expression in this blood or a cell is detected.

As a cell made applicable to detection, the cell ADIPUSHIN and its gene are carrying out [ the cell ] the high manifestation is desirable, and a fat cell and a liver cell are the most desirable.

[0042]

As the detection approach of an ADIPUSHIN gene, all RNA can be first extracted from the isolated blood or the cell, for example, and the approach of detecting the amount of manifestations of the ADIPUSHIN gene (mRNA) in [ this all ] RNA can be mentioned.

(1) The extract of all RNA

The extract of all RNA is extracted using the solvent for an RNA extract according to a well-known approach from the isolated blood or the cell. The things (for example, a TRIzol reagent: product made from Gibco BIAUERU etc.) containing the component which has the operation which inactivates RNase, such as a phenol, as this extracting solvent, for example are desirable. Especially the extract approach of RNA is not limited, for example,



can adopt thiocyanic acid guanidine and a cesium chloride ultracentrifugal method, a thiocyanic acid guanidine hot phenol process, a guanidine hydrochloric-acid method, the acid thiocyanic acid guanidine phenol chloroform method (Chomczynski, P. and Sacchi, N. (1987), *Anal. Biochem.*, 162, 156-159), etc. Especially, the acid thiocyanic acid guanidine phenol chloroform method is suitable.

[0043]

All extracted RNA may be further refined and used only for mRNA if needed. Although especially the purification approach is not limited, since it has the Pori (A) array in that three-dash terminal, many of mRNA(s) which exist in the cytoplasm of an eukaryotic cell can be carried out as follows, using this description. First, a biotin-ized oligo (dT) probe is added to all extracted RNA, and (Pori A) +RNA is made to adsorb. Next, the paramagnetism particle support which fixed streptoavidin is added and (Pori A) +RNA is made to catch using association between a biotin/streptoavidin. (Pori A) +RNA is eluted from an oligo (dT) probe at the last after washing actuation. (Pori A) +RNA may be made to adsorb using an oligo (dT) cellulose column besides this approach, and the approach of it being eluted and refining this may also be adopted. Fractionation of the eluted (Pori A) +RNA may be further carried out with a sucrose density-gradient centrifugation method etc.

[0044]

(2) Detection of an ADIPUSHIN gene

Next, the amount of ADIPUSHIN gene expression in [ all / under administration of an examined substance or the conditions of not prescribing a medicine for the patient ] RNA is detected. The amount of gene expression is detectable as the signal reinforcement by preparing cRNA or cDNA and carrying out the label of this with a suitable labeled compound from all obtained RNA.

[0045]

Hereafter, it divides into the analysis approach and the iRT-PCR method (real-time PCR method) which used i solid phase-ized sample, and the analysis approach of iii and others, and the detection approach of the amount of gene expression is explained concretely.

i) The analysis approach using a solid phase-ized sample

It mixes in the solid phase-ized sample which fixed the well-known gene, and it is made to hybridize separately simultaneously cDNA or cRNA (henceforth an "indicator probe") under administration or the conditions of not prescribing a medicine for the patient which carried out the indicator on the same conditions (Brown, P.O. et al. (1999) *Nature genet.* 21, suppliment, 33-37). That to which the mRNA clone of ADIPUSHIN also carried out the indicator of all the discovered mRNA(s) is sufficient as said indicator probe. Although mRNA which is not refined may be used as a start ingredient for probe

production, it is more desirable to use (Pori A) +RNA refined by the above-mentioned approach. Hereafter, the analysis approach using various solid phase-ized samples is explained.

[0046]

a) Gene chip :

As long as the ADIPUSHIN gene which is an object for detection is solid-phase-ized, the gene chip used by this invention may be produced based on a well-known approach (Lipshutz, R.J. et al.(1999) Nature genet.21, suppliment, 20-24), even if it is a commercial thing. For example, as that by which the mouse ADIPUSHIN gene was fixed, mouse MG-U74 (U74A, U74B, U74C) by the AFI metrics company etc. can be mentioned.

Detection by the gene chip can be carried out according to a conventional method. For example, if it is the case where the chip by the AFI metrics company is used, according to the protocol attached to the product, the cRNA probe which carried out the biotin indicator will be prepared.

Subsequently, hybridization is performed according to this protocol, and if luminescence by avidin is detected and analyzed, the amount of gene expression can be calculated.

[0047]

b) An array or membrane filter :

As long as the ADIPUSHIN gene which is an object for detection is solid-phase-ized, the array or membrane filter used by this invention may be produced based on a well-known approach, even if it is a commercial thing (for example, intelligent gene:TAKARA SHUZO [ CO., LTD. ] make, an atlas system: Clonetec make etc.). cDNA or the RT-PCR product cloned by carrying out a reverse transcriptase reaction and PCR by the primer produced based on array information, such as GenBank, is used for the gene solid-phase-ized.

[0048]

In the detection using an array, in case (Pori A) +RNA to cDNA is produced at a reverse transcriptase reaction, an indicator probe is prepared by adding d-UTP by which the indicator was carried out by the fluorochrome (for example, Cy3, Cy5 grade). If the indicator of (Pori A) +RNA under administration of an examined substance and the (Pori A) +RNA under un-prescribing [ of an examined substance ] a medicine for the patient is carried out with coloring matter different, respectively at this time, both can be mixed and used at the time of next hybridization. A fluorescence signal is detected using a fluorescence signal detection machine. For example, if it is the commercial array of TAKARA SHUZO CO., LTD., according to the protocol of the company, hybridization and washing will be performed, and a fluorescence signal will be detected and analyzed with fluorescence signal detection machines (for example, a GMS418 array scanner: TAKARA SHUZO

CO., LTD. make etc.).

[0049]

In the detection using a membrane filter, in case (Pori A) +RNA to cDNA is produced at a reverse transcriptase reaction, by adding d-CPT by which the indicator was carried out with radioisotope (for example,  $^{32}\text{P}$ ,  $^{33}\text{P}$ ), an indicator probe is prepared and hybridization is performed with a conventional method. For example, after performing hybridization and washing using the atlas system (Clonetec make) which is a commercial microarray made from a filter, it analyzes by detecting using analysis equipments (for example, an atlas image: Clonetec make etc.).

[0050]

Also when using which solid phase-ized sample, administration of an examined substance or the probe under the conditions of not prescribing a medicine for the patient is made to hybridize, respectively, and a difference of the amount of gene expression is detected. At this time, hybridization conditions other than the probe to be used are made the same. A difference of the amount of gene expression is detectable by making one solid phase-ized sample hybridize the mixture of both probes at once, and reading fluorescence intensity, if the indicator of each probe is carried out by different fluorochrome in the case of the fluorescent-labeling probe as mentioned above (Brown, P.O. et al.(1999) Nature genet.21, suppliment, 33-37).

[0051]

ii) RT-PCR method (real-time PCR method)

the real time PCR (TaqMan PCR) which is RT-PCR method and its one -- law is suitable for the assessment approach of this invention in minute amount DNA at high sensitivity and the point of being quantitatively detectable. real time PCR (TaqMan PCR) -- law -- 5' edge -- a fluorochrome (reporter) -- 3' -- the oligonucleotide probe which hybridizes an edge to the specific region of the object gene which carried out the indicator by the fluorochrome (quencher) is used. As for this probe, a reporter's fluorescence is controlled by the quencher in the usual condition. In the condition of having made the object gene hybridizing this fluorescent probe thoroughly, it is that outside to Taq. PCR is performed using DNA polymerase. Taq If the expanding reaction by DNA polymerase progresses, a fluorescent probe will be hydrolyzed by the exonuclease activity from 5' edge, reporter coloring matter will separate, and fluorescence will be emitted. The real-time PCR method can carry out the quantum of the primary quantity of template DNA to accuracy by carrying out monitoring of this fluorescence intensity on real time.

[0052]

For example, if it is the case of this invention, the probe (for example, probe

which consists of a base sequence shown in the array number 6) for detecting specifically the primer (for example, primer which consists of a base sequence shown in the array number 4 and the array number 5) which amplifies a mouse ADIPUSHIN gene (mRNA) specifically, and a mouse ADIPUSHIN gene will be designed, and real time PCR (TaqMan PCR) will be performed. If the amount of ADIPUSHIN gene expression under the administration conditions of an examined substance is increasing more remarkably than the bottom of the condition of not prescribing a medicine for the patient, it can be estimated that this examined substance has an insulin resistance improvement effect.

[0053]

iii) The other analysis approaches

As an approach of analyzing the amount of gene expression in addition to the above For example Subtraction technique () [ Sive, ] [ H.L.] and John and T.St. (1988) Nucleic Acids Research 16, 10937, Wang, Z., and Brown, D.D.(1991) Proc.Natl.Acad.Sci.U.S.A.88, 11505-11509, The differential displaying method (it Liang(s)) P., and Pardee, and A.B. Science 257, (1992) 967-971, Liang, P., Averboukh, L., Keyomarsi, K., Sager, R., and Pardee, A.B.(1992) Cancer Research 52, 6966-6968, A differential hybridization method (John and T.St., and Davis, R.W.Cell (1979) 16, 443-452), moreover A suitable probe The used cross hybridization method () [ "Molecular Cloning and A Laboratory Manual" Maniatis, T., Fritsch, E.F., Sambrook, ] [ J.] (1982) Cold Spring Harbor Laboratory Press etc. can be mentioned. When the above-mentioned approach examines collectively other gene expression profiles specifically discovered under the administration conditions of an examined substance in addition to an ADIPUSHIN gene, it is useful.

[0054]

a) Subtraction cloning process :

It is the approach of carrying out cloning of the gene by acquiring cDNA of the gene specifically discovered into a specific cell, and screening a cDNA library by using this cDNA as a probe. As the approach of subtraction, a single strand cDNA is produced from all RNA. After making all RNA obtained from the cell different from this hybridize, the single stranded DNA which was not hybridized in a hydroxyapatite column is isolated. the approach (the biotechnology manual series 3 and a gene-cloning laboratory procedure --) of producing the cDNA library from this cDNA Yodosha (1993) Current PUROTO call Inn molecular biology, Produce a cDNA library first and a single stranded DNA is prepared using a helper phage etc. from this library. After making what carried out the biotin indicator to all RNA obtained from the cell different from this single stranded DNA hybridize, The single stranded DNA which was not hybridized using avidin is isolated. By DNA polymerase It returns to a double strand. A cDNA library How to produce () [ Tanaka, H.,

Yoshimura, Y., Nishina, Y., Nozaki, M., Nojima, H., and Nishimune ] [ Y.(1994) FEBS ] Lett.355, 4-10, etc. are mentioned.  
[0055]

All RNA that refined mRNA or all RNA first about administration of an examined substance or each specimen under the conditions of not prescribing a medicine for the patient, and was specifically refined from the specimen under administration conditions is used as mold, and cDNA is compounded with reverse transcriptase. The indicator of the cDNA can also be carried out by adding [ $\alpha$ - $^{32}$ P] dNTP at the time of composition. Although all RNA used as cDNA by which the indicator was carried out, and mold forms the stable double-stranded-DNA-RNA hybrid, by carrying out high temperature processing under alkali existence, it decomposes only RNA and generates a single strand cDNA. If this single strand cDNA and RNA extracted from the specimen under the conditions of not prescribing a medicine for the patient are mixed and it puts under suitable conditions, a double-stranded-DNA-RNA hybrid more stable than the complementarity of a nucleotide sequence will be formed. That is, although cDNA which uses as mold all RNA discovered also under the conditions of not prescribing a medicine for the patient forms a hybrid, cDNA which used as mold RNA specifically discovered only under administration conditions is still a single strand. Subsequently, a hydroxyapatite column separates a double-stranded-DNA-RNA hybrid and a single strand cDNA, and a single strand cDNA is refined. Specific cDNA can be condensed by repeating this step in the organization which considered as the object. Condensed specific cDNA can be used as a probe which screens a cDNA library, when the indicator is carried out with radioisotope etc. In addition, this actuation can also be performed using commercial kits (for example, an PCR selection cDNA subtraction kit: Clonetec make etc.).

[0056]

b) The differential displaying method :

According to Liang's and others approach (Science (1992) 257, 967-971), it can carry out as follows. mRNA or all RNA is extracted from two samples (in the case of this invention, they are administration of an examined substance, or a specimen under the conditions of not prescribing a medicine for the patient) compared first, and this is changed into a single strand cDNA using reverse transcriptase. Subsequently, PCR is performed by using the obtained single strand cDNA as mold using a suitable primer. As a primer, a random primer (primer of about ten to 12 mer which consists of an array of arbitration) can be used, for example. Or you may use combining support Doppler IMA (anchored primer) and every one sort each of ABITO rally primers (arbitrary primer). as support Doppler IMA -- oligo d (T) -- the primer which consists of nVX [n= 11 - a 12; V= guanine, an adenine or a

cytosine;X= guanine, an adenine, a thymine, or a cytosine] can be used. Moreover, as an ABITO rally primer, the random primer of about 10 mer(s) which consists of an array of arbitration can be used. It becomes possible to screen the gene cluster of the larger range by performing such PCR combining various primers. Then, by carrying out gel electrophoresis of the acquired PCR product, and carrying out comparison analysis of the manifestation pattern (fingerprints) of all RNA developed on gel (display), the gene (ADIPUSHIN gene) specifically discovered by one of specimens can be chosen, and the cDNA fragment can be isolated. In addition, this approach can also be performed using the kits (for example, an RNA image kit: JIEN hunter company make etc.) marketed.

[0057]

c) Differential hybridization method :

The cDNA library produced from all RNA refined from the target organization is screened with the 32P indicator cDNA probe compounded from all RNA of the object organization and a contrast organization, and they are the probe of the object organization, and the approach of choosing the clone which hybridizes. For example, according to a conventional method, a cDNA library is produced from all RNA first refined from the specimen under the conditions of not prescribing a medicine for the patient, and 2 sets of replica filters are produced from the library. Next, cDNA is compounded with reverse transcriptase by using as mold all RNA refined from the specimen under these conditions of not prescribing a medicine for the patient. The indicator of the cDNA is carried out by adding [ $\alpha$ -32P] dNTP at the time of composition. Although all RNA used as cDNA by which the indicator was carried out, and mold forms the stable double-stranded-DNA-RNA hybrid, by carrying out high temperature processing under alkali existence, only all RNA is decomposed and a single strand cDNA is refined. The single strand cDNA by which similarly the indicator was carried out to mold by 32P in all RNA refined from the specimen under examined substance administration conditions is produced. The filter and hybridization which were produced from the specimen under the conditions of not prescribing a medicine for the patient are performed by using both the indicators cDNA as a probe, respectively. The autoradiography image of an X-ray film is compared and cloning of the gene (ADIPUSHIN gene) specifically discovered under the administration conditions of an examined substance can be carried out by choosing the clone hybridized only to one side of the cDNA probe under administration or the conditions of not prescribing a medicine for the patient.

[0058]

d) Cross hybridization method :

Hybridization is performed on the conditions that stringency is low, by using

suitable DNA as a probe to the cDNA library originating in either administration of an examined substance, or the specimen under the conditions of not prescribing a medicine for the patient, and an electropositive clone is obtained. Northern hybridization is performed to all RNA originating in each specimen by using the obtained electropositive clone as a probe, and the clone discovered only to one side is chosen. In this way, it can check that performed Northern blotting by having used obtained cDNA as the probe to all RNA of the specimen under administration or the conditions of not prescribing a medicine for the patient, and all RNA of the selected gene (ADIPUSHIN gene) is specifically discovered under administration conditions.

[0059]

Process 3: Assessment of an insulin resistance improvement effect  
At the last, the insulin resistance improvement effect of this examined substance is evaluated based on a difference of the amount of manifestations of ADIPUSHIN in administration or un-prescribing a medicine for the patient. [ of an examined substance ]

That is, when the amount of ADIPUSHIN gene expression is increasing from under the condition of not prescribing a medicine for the patient, intentionally under the administration conditions of an examined substance, it can be estimated that this examined substance has an insulin resistance improvement effect. Here, it means that statistical significance ( $p < 0.05$ ) is in the amount of ADIPUSHIN gene expression under administration of an examined substance, and the conditions of not prescribing a medicine for the patient, saying "it is increasing intentionally."

[0060]

## 2.2 Assessment Approach of Examined Substance Which Made ADIPUSHIN Index (Inch Vivo)

As for assessment of the insulin resistance improvement effect of the examined substance in in vivo which made ADIPUSHIN the index, it is desirable that it is an approach including the following process.

Process 1: Breed an animal under the conditions of administration of an examined substance, or not prescribing a medicine for the patient.

Process 2: Detect the blood of the above-mentioned animal, or the amount of manifestations of ADIPUSHIN in a cell using the antibody specifically combined with this ADIPUSHIN.

Process 3: Evaluate the insulin resistance improvement effect of this examined substance based on a difference of the amount of manifestations of ADIPUSHIN in administration or un-prescribing a medicine for the patient. [ of an examined substance ]

[0061]

Process 1: Breeding of an animal



An animal is bred according to the approach indicated for the preceding clause 2.1 under the conditions of administration of an examined substance, or not prescribing a medicine for the patient.

Process 2: Detection of the amount of ADIPUSHIN manifestations

Next, the blood of the above-mentioned animal or the amount of manifestations of ADIPUSHIN in a cell is detected using the antibody specifically combined with this ADIPUSHIN.

[0062]

Although especially the detection approach of ADIPUSHIN using an antibody is not limited, it is desirable that it is any 1 approach chosen from a Western blot technique, dot blotting methods, slot blotting methods, the ELISA method, and the RIA method. Hereafter, from preparation of a sample to detection is concretely explained about these detection approaches.

(1) Preparation of a sample

As a specimen, the cell blood, or ADIPUSHIN and its gene are carrying out [ the cell ] the high manifestation is desirable, and a fat cell and a liver cell are the most desirable. These blood or cells (it is used as a cell extract) are prepared as the sample for ELISA/RIA, or a sample for western blotting as follows, after removing the insoluble matter by performing high-speed centrifugal one if needed.

What was suitably diluted with the buffer solution, for example, using the collected blood serum as it is is used for the sample for ELISA/RIA. The sample for western blotting (for electrophoresis) is suitably diluted with the buffer solution, using for example, a cell extract as it is, and what was mixed with the sample buffer solutions (sigma company make etc.) containing 2-Mercator ethanol for SDS-polyacrylamide gel electrophoreses is used. The thing which the collected cell extract itself or the thing suitably diluted with the buffer solution was used [ thing ], and made it adsorb blotting equipment to a direct membrane for example is used for a dot / sample for slot blots.

[0063]

(2) Solid-phase-izing of a sample

By the above-mentioned approach, the polypeptide in the sample in which ADIPUSHIN is contained is first solid-phase-ized on the base in a well of a membrane or 96 hole plate etc.

The approach (western blotting) of imprinting a polypeptide to a membrane through the polyacrylamide gel electrophoresis of a sample as an approach of solid-phase-izing to a membrane and the method (dot blotting methods and slot blotting methods) of infiltrating a sample or its diluent into a direct membrane can be mentioned. As a membrane used, nitrocellulose membranes (for example, Bio-Rad make etc.), nylon membranes (for example, yes bond-ECL (Amersham Pharmacia manufacture) etc.), cotton membranes (for example, blot ABUSO vent filter (Bio-Rad make) etc.), or the poly vinylidene



JIFURUORIDO (PVDF) membranes (for example, Bio-Rad make etc.) can be mentioned. Moreover, as the blotting approach, the wet type blotting method (CURRENT PROTOCOLS IN IMMUNOLOGY volume 2 ed by J.E.Coligan, A.M.Kruisbeek, D.H.Margulies, E.M.Shevach, W.Strober), the semi dry type blotting method (above-mentioned CURRENT PROTOCOLS IN IMMUNOLOGY volume 2 reference), etc. can be mentioned.

[0064]

On the other hand, as an approach of solid-phase-izing, solid phase enzyme immunoassay (the ELISA method), radioisotope immunoassay (the RIA method), etc. can be mentioned to 96 hole plate. solid-phase-izing -- for example, said 96 hole plates (for example, immuno plate maxi soap (Nunc make) etc.) -- a sample or its diluent (for example, thing diluted with the phosphate buffered saline (henceforth "PBS") which contains a sodium azide 0.05%) -- putting in -- 4 degrees C -- a room temperature -- a night or 37 degrees C -- 1 - 3 hours -- putting -- a well -- what is necessary is just to make a polypeptide stick to a base

[0065]

(3) The antibody specifically combined with ADIPUSHIN (anti-ADIPUSHIN antibody)

"The antibody (henceforth a "anti-ADIPUSHIN antibody") specifically combined with ADIPUSHIN" used at this process may be prepared according to a well-known approach, and commercial things (for example, the anti-mouse ADIPUSHIN antibody p-16, the product made from St. KURUZU, etc.) may be used for it.

[0066]

Said antibody can carry out immunity of the animal using the polypeptide of ADIPUSHIN used as an antigen, or the arbitration chosen from the amino acid sequence with a conventional method (397 for example, the new chemistry experiment lecture 1, protein 1, p.389- 1992), and can obtain it by extracting and refining the antibody produced in this animal living body. Moreover, by uniting the antibody forming cell and myeloma cell which produce the antibody to ADIPUSHIN of this invention according to a well-known approach (for example, Kohler and Milstein, Nature 256, 495-497, 1975, Kennet, and R.ed., Monoclonal Antibody p.365-367, 1980, Prenum Press, N.Y.), a hybridoma can be established and a monoclonal antibody can also be obtained from this.

[0067]

As an antigen for antibody production, the polypeptide which consists of ADIPUSHIN of this invention or its at least six continuous partial amino acid sequence, or the derivative with which the amino acid sequence and support of arbitration were added to these can be mentioned. Especially, what combined keyhole limpet hemocyanin with the amino terminal of ADIPUSHIN

of this invention as support is desirable.

[0068]

Said antigen polypeptide can be obtained by making a host cell produce ADIPUSHIN of this invention by genetic manipulation. What is necessary is to produce the vector in which the ADIPUSHIN gene expression of this invention is possible, to specifically introduce this into a host cell, and just to make this gene discover.

[0069]

As said host cell, if it is a prokaryotic cell, *Escherichia coli* (*Escherichia coli*), a *Bacillus subtilis* (*Bacillus subtilis*), etc. will be mentioned, for example. In order to carry out the transformation of the target gene by these host intracellular, the transformation of the host cell is carried out by the plasmid vector containing a regulatory sequence, the replicon of the seed origin, i.e., the replication origin, which may suit with a host. What has the array which can give the selectivity of the quality of a phenotype (phenotype) to a transformed cell as this vector is desirable.

[0070]

For example, although K12 share etc. will be used well and the plasmid of a pBR322 and pUC system will generally be used as a vector if it is *Escherichia coli*, it is not limited to these but various well-known strain and a well-known vector can be used. Moreover, as a promotor used with *Escherichia coli*, a tryptophan (*trp*) promotor, a lactose (*lac*) promotor, a tryptophan lactose (*tac*) promotor, a lipoprotein (*lpp*) promotor, a polypeptide chain extension factor Tu (*tufB*) promotor, etc. can be mentioned, and all can be used suitably, for example.

[0071]

Moreover, although 207 to 25 shares will be desirable and pTUB228 (Ohmura, K. et al.(1984) *J.Biochem.*95, 87-93) etc. will be used as a vector if it is a *Bacillus subtilis*, it is not limited to this. In addition, a secretion manifestation out of a biomass is also attained by connecting the DNA array which carries out the code of the transit peptide array of the alpha-amylase of a *Bacillus subtilis* to a vector.

[0072]

Cells, such as a vertebrate, an insect, and yeast, are mentioned as a host cell of an eukaryotic cell. the COS cell (Gluzman, Y.(1981) *Cell* 23, 175-182, ATCC CRL-1650) which is a cell of an ape as a vertebrate cell, for example, and a Chinese hamster ovary cell (a CHO cell —) Although the dihydroleaf redox enzyme defect stock (Urlaub, G. and Chasin, L.A.(1980)

*Proc.Natl.Acad.Sci.USA* 77, 4126-4220) of ATCC CCL-61 etc. is used well It is not limited to these.

[0073]

What has the promotor located in the upstream of the gene which you are

going to make it usually discover as an expression vector of a vertebrate cell, the splice site of RNA, a polyadenylation part, a termination array of an imprint, etc. can be used. Furthermore, this may have a replication origin as occasion demands. Although pSV2dhfr (Subramani, S. et al. (1981) Mol. Cell. Biol. 1, 854-864) which has pCR3.1 (product made from Invitrogen) and the initial promotor of SV40 who have the initial promotor of a cytomegalovirus is mentioned as an example of this expression vector, it is not limited to these.

[0074]

If the case where a COS cell is used is mentioned as an example as a host cell, as an expression vector, it has an SV40 replication origin, and in a COS cell, independence growth is possible, and a transcriptional promoter, the termination signal of an imprint, and the thing equipped with the RNA splice site can be used further suitably. This expression vector The diethylaminoethyl (DEAE)-dextran method (Luthman, H. and Magnusson, G. (1983) Nucleic Acids Res, 11, 1295-1308), A calcium phosphate-DNA coprecipitation Mr. method (Graham, F. L. and van der Eb, A. J. (1973) Virology 52, 456-457), And a COS cell can be made to be able to incorporate by the electric pulse terebration (Neumann, E. et al. (1982) EMBO J. 1, 841-845) etc., and a desired transformed cell can be obtained in this way.

[0075]

moreover, in using a CHO cell as a host cell The vector which may discover the neo gene which functions as an antibiotic G418 resistance marker with an expression vector, For example pRSVneo () [ Sambrook, ] [ J. ] et al. (1989): "Molecular Cloning A Laboratory Manual" Cold Spring Harbor Laboratory, NY, pSV2neo (Southern, P. J. and Berg, P. (1982) J. Mol. Appl. Genet. 1, 327-341), etc. are KO and transfected. By choosing the colony of G418 resistance, the transformed cell which produces the target polypeptide to stability can be obtained.

[0076]

In using an insect cell as a host cell The ovarian cell origin established cell line (Sf-9 or Sf-21) of *Spodoptera frugiperda* of *Lepidoptera Phalaenidae*, and the ootid origin High Five cell of *Trichoplusia ni* () [ Wickham, ] [ T. J. ] et al (1992), Biotechnol. Prog. i: 391-396 etc. is well used as a host cell. As a baculovirus transfer vector pVL 1392/1393 using the promotor of the PORIHE drine compounds protein of a \*\* *Autographa nuclear polyhedrosis virus* (AcNPV) is used well (). [ Kidd, ] [ i. M. and V. C. Emery (1993) The ] use of baculoviruses as expression vectors. Applied Biochemistry and Biotechnology 420, 137-159. In addition, the vector using the promotor of the P10 and this basic protein of a baculovirus can also be used. Furthermore, it is possible by tying the secretion signal array of the envelope surface protein GP67 of AcNPV to the amino terminal side of the object protein to also

make recombination protein discover as secretory protein (Zhe-mei Wang, et al.(1998) Biol.Chem., 379, 167-174).

[0077]

As a manifestation system which made the eukaryon microorganism the host cell, generally yeast is known well and *Saccharomyces* yeast, for example, baker's yeast *Saccharomyces cerevisiae* and petroleum yeast *Pichia pastoris*, is desirable also in it. As an expression vector of eukaryon microorganisms, such as this yeast For example The promotor of an alcoholic dehydrogenase gene () [ Bennetzen, ] [ J.L.and ] Hall and B.D.(1982) J. Biol.Chem.257, 3018-3025, the promotor (Miyanohara, A.et al.(1983) Proc.Natl.Acad.Sci.USA 80, 1-5) of an acid phosphatase gene, etc. It can use preferably. Moreover, when making it discovered as secretor protein, it is possible to also make it discovered as recombinant which has at least the cutting section of the internality protease or the known protease which a secretion signal array and a host cell have in an amino terminal side. for example, by the system made to discover with petroleum yeast, *Homo sapiens* mast cell TORIPUTAZE of a trypsin mold serine protease By carrying out the bond manifestation only of the cutting section of KEX2 protease which the secretion signal array of alpha factor of yeast and petroleum yeast have in an amino terminal side It is known that active TORIPUTAZE will be secreted in a culture medium (Andrew, L.Niles, et al.(1998) Biotechnol.Appl.Biochem.28, 125-131).

[0078]

The transformant obtained as mentioned above can be cultivated according to a conventional method, and the target polypeptide is produced by this culture out of intracellular or a cell. Various kinds of culture media commonly used as a culture medium used for this culture according to the adopted host cell can be chosen suitably. For example, if it is the above-mentioned COS cell, what added blood serum components, such as fetal calf serum, if needed to culture media, such as RPMI1640 culture medium and an Dulbecco's modified Eagle's medium (henceforth "DMEM"), can be used.

[0079]

The recombination protein produced out of intracellular [ of a transformant ] or a cell by the above-mentioned culture can be separated and refined by the well-known separation operation information using a physical property, chemical property, etc. of this protein. independent [ in various liquid chromatography, such as processing according to a protein precipitant for example, an ultrafiltration, a molecular sieve chromatography (gel filtration), adsorption chromatography, an ion exchange chromatography, affinity chromatography, and high performance chromatography (HPLC) and dialysis ] as this approach -- or it can combine and use. Moreover, if the histidine which becomes the recombination protein made to discover from 6 residue is connected, it can refine efficiently by the nickel affinity column. The target

ADIPUSHIN protein can be easily manufactured by high yield and the high grade by combining suitably the approach indicated above.

[0080]

#### (4) Detection

The anti-ADIPUSHIN antibody obtained by the approach of the above-mentioned (3) publication carries out the direct indicator of it, or makes this antibody a primary antibody, and is used for detection in cooperation with the indicator (antibody of the animal origin which produced antibody is recognized) second antibody which recognizes this primary antibody specifically.

[0081]

Although a thing desirable as a class of said indicator is an enzyme (alkaline phosphatase or horseradish peroxidase) or a biotin (however, actuation of combining enzyme-labeling streptoavidin with the biotin of a second antibody further is added), it is not limited to these. As an indicator second antibody (or indicator streptoavidin), various marketing of the antibody (or streptoavidin) by which the indicator was carried out beforehand is carried out. In addition, measurement is performed using a liquid scintillation counter etc. using the antibody to which the indicator of the case of RIA was carried out with radioisotope, such as  $^{125}\text{I}$ .

[0082]

By detecting the activity of these enzymes by which the indicator was carried out, the amount of manifestations of ADIPUSHIN which is an antigen is measured. When carrying out an indicator by the alkaline phosphatase or horseradish peroxidase, the substrate colored according to the catalyst of these enzymes and the substrate which emits light are marketed.

[0083]

It is visually detectable, if western blotting, and a dot / slot blotting methods are used when the substrate to color is used. It is desirable to measure and carry out the quantum of the absorbance (for measurement wavelength to change with substrates) of each well by the ELISA method using a commercial microplate reader. Moreover, it is also possible to carry out the quantum of the antigen concentration in other samples by preparing the dilution sequence of the antigen used for above-mentioned antibody production, performing detection actuation to other samples and coincidence by making this into a standard antigen sample, and creating the standard curve which plotted standard antigen concentration and measured value.

[0084]

On the other hand, when the substrate which emits light is used, in western blotting, or a dot / slot blotting methods, the autoradiography using an X-ray film or an imaging plate and the photography using an instant camera can detect. Moreover, the quantum using a densitometry, a molecular imager Fx

system (Bio-Rad make), etc. is also possible. Furthermore, when using a luminescence substrate by the ELISA method, enzyme activity is measured using luminescence microplate readers (for example, Bio-Rad make etc.).

[0085]

(5) Measurement actuation

a) In the case of western blotting, a dot blot, or a slot blot

First, in order to prevent the nonspecific adsorption of an antibody, actuation (blocking) dipped fixed time into the buffer solution containing the matter (skim milk, casein, bovine serum albumin, gelatin, polyvinyl pyrrolidone, etc.) which checks such nonspecific adsorption for a membrane beforehand is performed. The presentation of a blocking solution is 5%. Skim milk and 0.05 – 0.1% The phosphate buffered saline (PBS) or the tris buffered saline solution (TBS) containing Tween 20 is used. Instead of skim milk, the block ace (Dainippon Pharmaceutical), 1 – 10% of bovine serum albumin, 0.5 – 3% of gelatin, or 1% of polyvinyl pyrrolidone may be used. The time amount of blocking is 1 – 3 hours at 16 – 24 hours, or a room temperature in 4 degrees C.

[0086]

Next, it is a membrane 0.05 – 0.1% After washing in PBS or TBS (henceforth a "penetrant remover") containing Tween 20 and removing an excessive blocking solution, an anti-ADIPUSHIN antibody is dipped fixed time into the solution suitably diluted with the blocking solution, and this antibody is combined with the antigen on a membrane. The dilution scale factor of the antibody at this time can be determined by conducting the preliminary Western-blotting experiment which made the sample what carried out phase dilution of said recombination antigen, for example. This antibody reaction actuation is preferably performed at a room temperature for 2 hours. A membrane is washed by the penetrant remover after antibody reaction actuation termination. Here, when the indicator of the used antibody is carried out, detection actuation can be performed immediately. When the antibody of a non-indicator is used, a second antibody reaction is performed succeedingly. An indicator second antibody is diluted and used 2000 to 20000 times with a blocking solution, when using a commercial thing (the publication is followed when the suitable dilution scale factor for attached instructions is indicated). After dipping the membrane after carrying out washing clearance of the primary antibody in a second antibody solution at a room temperature for 45 minutes to 1 hour and washing by the penetrant remover, detection actuation doubled with the indicator approach is performed. Washing actuation is performed by exchanging penetrant removers again and shaking for 5 minutes, after shaking a membrane for 15 minutes in a penetrant remover first, exchanging a penetrant remover for a new thing and shaking it for 5 minutes for example. A penetrant remover may be exchanged and

washed further if needed.

[0087]

b)ELISA/RIA

First, in order to prevent the nonspecific adsorption of the antibody to the base in a well of the plate which made the sample solid-phase-ize, it blocks beforehand like the case of western blotting. It is as having indicated the conditions of blocking in the term of western blotting.

[0088]

Next, it is the inside of a well 0.05 – 0.1% After washing in PBS or TBS (henceforth a “penetrant remover”) containing Tween 20 and removing an excessive blocking solution, a fixed time amount incubation of the anti-ADIPUSHIN antibody suitably diluted with the penetrant remover is poured distributively and carried out, and this antibody is combined with an antigen. The dilution scale factor of the antibody at this time can be determined by conducting the preliminary ELISA experiment which made the sample what carried out phase dilution for example, of the above-mentioned recombination antigen. This antibody reaction actuation is preferably performed at a room temperature for about 1 hour. After antibody reaction actuation termination and the inside of a well are washed by the penetrant remover. Here, when the indicator of the used antibody is carried out, detection actuation can be performed immediately. When the antibody of a non-indicator is used, a second antibody reaction is performed succeedingly. An indicator second antibody is diluted and used 2000 to 20000 times by the penetrant remover, when using a commercial thing (the publication is followed when the suitable dilution scale factor for attached instructions is indicated). After pouring a second antibody solution distributively to the well after carrying out washing clearance of the primary antibody, carrying out an incubation at a room temperature for 1 to 3 hours and washing by the penetrant remover, detection actuation doubled with the indicator approach is performed. Washing actuation is performed by exchanging penetrant removers again and shaking for 5 minutes, after pouring a penetrant remover distributively, shaking for 5 minutes in a well first for example, exchanging a penetrant remover for a new thing and shaking it for 5 minutes. A penetrant remover may be exchanged and washed further if needed.

[0089]

For example, in this invention, the so-called ELISA of a sandwich technique can be carried out by the approach of indicating below. First, two fields which are rich in a hydrophilic property are chosen from each amino acid sequence of the ADIPUSHIN protein of this invention, respectively. Next, the partial peptide which consists of 6 or more residue of amino acid in each field is compounded, and two kinds of antibodies which used this partial peptide as the antigen are acquired. Among these, the indicator of one antibody is



carried out. The antibody of the direction which did not carry out an indicator is solid-phase-ized on the base in a well of the plate for 96 hole ELISA. After blocking, a sample solution is paid in a well and an incubation is carried out in ordinary temperature for 1 hour. The incubation of the antibody diluent of the direction which carried out the indicator is poured distributively and carried out to each well after washing the inside of a well. Detection actuation doubled with the indicator approach is performed after washing the inside of a well again.

[0090]

Process 3: Assessment of an insulin resistance improvement effect

At the last, the insulin resistance improvement effect of this examined substance is evaluated based on a difference of the amount of manifestations of ADIPUSHIN in administration or un-prescribing a medicine for the patient. [ of an examined substance ]

That is, when the amount of manifestations of ADIPUSHIN is increasing from under the condition of not prescribing a medicine for the patient, intentionally under the administration conditions of an examined substance, it can be estimated that this examined substance has an insulin resistance improvement effect. Here, it means that statistical significance ( $p < 0.05$ ) is in administration of an examined substance, and the amount of manifestations of ADIPUSHIN under the conditions of not prescribing a medicine for the patient, saying "it is increasing intentionally."

[0091]

2.3 Assessment Approach of Examined Substance Which Made ADIPUSHIN or ADIPUSHIN Gene Index (Inch Vitro)

As for the assessment approach of the insulin resistance improvement effect of the examined substance in in vitro which made ADIPUSHIN or an ADIPUSHIN gene the index, it is desirable to include the following process.

Process 1: Cultivate a cell under administration of a specimen material, or the conditions of not prescribing a medicine for the patient.

Process 2: Detect the amount of ADIPUSHIN gene expression in the above-mentioned cell, or detect the amount of manifestations of ADIPUSHIN using the antibody specifically combined with this ADIPUSHIN.

Process 3: Evaluate the insulin resistance improvement effect of this examined substance based on a difference of the above-mentioned ADIPUSHIN gene under administration of a specimen material or the conditions of not prescribing a medicine for the patient, or the amount of manifestations of ADIPUSHIN.

[0092]

Process 1: Culture of a cell

The cell used by the assessment approach of this invention will not be limited especially if it is the mammalian cell which discovers an ADIPUSHIN



gene. The cultured cell (preferably primary culture hepatocyte) of the mammalian origin, especially the liver origin of mammalian is preferably desirable. As said mammalian, Homo sapiens, a mouse, a rat, or a hamster is desirable, and Homo sapiens or a mouse is more desirable. Especially, as a suitable example, although the primary culture hepatocyte of the type 2 diabetes model animal (for example, above-mentioned KK mouse etc.) origin can be mentioned, it is not limited to these.

Moreover, cells (for example, CHO cell) by which the transformation was carried out artificially, such as a cell which introduced the ADIPUSHIN gene with the promoterregion, may be used.

[0093]

A cell cultivates a specimen material under administration or the conditions of not prescribing a medicine for the patient. Especially the culture approach is not limited but should just choose the culture approach suitable for the cell concerned suitably. What is necessary is to also limit neither the administration (addition) approach of the specimen material to a cultured cell, nor especially a dose, but to add a specimen material to a culture culture medium, and just to carry out fixed period culture during culture of the above-mentioned cell. Although what is necessary is just to also set up suitably the period cultivated under specimen material existence, it is 30 minutes – 24 hours preferably.

[0094]

Process 2: Detection of ADIPUSHIN or the amount of ADIPUSHIN gene expression

Next, a difference of the amount of ADIPUSHIN gene expression in administration of a specimen material or the above-mentioned cell under the conditions of not prescribing a medicine for the patient is detected, or a difference of the amount of manifestations of ADIPUSHIN is detected using the antibody specifically combined with this ADIPUSHIN.

[0095]

What is necessary is just to perform detection of an ADIPUSHIN gene according to the approach fundamentally indicated to 2.1. Moreover, what is necessary is just to perform detection of ADIPUSHIN using an anti-ADIPUSHIN antibody according to the approach indicated to 2.2.

[0096]

ADIPUSHIN and ADIPUSHIN gene expression are also indirectly detectable under promotor rule of an ADIPUSHIN gene besides the above-mentioned approach using the gene (henceforth a "reporter gene") which enables detection of this promotor activity. Hereafter, the detection approach using a reporter gene is explained.

(1) Reporter gene

A reporter gene should just carry out the code of the distinguishable

reporter protein to any protein of the others which a host cell can produce in a series of processes of the exam approach specifically. That desirable the cell before a transformation is the same as that of this reporter protein or a desirable thing which does not have the gene which carries out the code of the similar protein is good. For example, even when it is that in which reporter protein has toxicity to this cell, and the thing which this cell gives the resistance of the antibiotic which has susceptibility, the existence of a manifestation of a reporter gene can be judged with the survival rate of a cell. However, the more desirable thing as a reporter gene used by this invention is the structural gene (as [ acquire / for example, / the specific antibody to the protein by which a code is carried out to this reporter gene ]) which can detect the amount of manifestations specifically and quantitatively. It is the gene which carries out the code of the enzyme with which quantitative measurement produces easy metabolite by reacting specifically with a foreign substrate more preferably. As such a reporter gene, although the gene which carries out the code of the following protein can be illustrated, this invention is not limited to them, for example.

[0097]

a) Chloramphenicol acetyltransferase :

It is detectable by the so-called CAT assay etc. with the enzyme which adds an acetyl group to a chloramphenicol. As a vector which can prepare the vector for reporter assays only by incorporating a promotor, the pCAT3-Basic vector (pro megger company make) is marketed.

b) Firefly luciferase :

A quantum can be carried out by measuring the bioluminescence produced when luciferin is metabolized. As a vector for reporter assays, the pGL3-Basic vector (pro megger company make) is marketed.

c) Beta-galactosidase :

There is a respectively measurable substrate by color reaction, fluorescence, or chemiluminescence. As a vector for reporter assays, pbetagal-Basic (pro megger company make) is marketed.

d) Secretor alkaline phosphatase :

There is a respectively measurable substrate by color reaction, the bioluminescence, or chemiluminescence. pSEAP2-Basic (Clontech make) is marketed as a vector for reporter assays.

e) Green fluorescence protein (green-fluorescent protein) :

Although it is not an enzyme, since oneself emits fluorescence, a direct quantum can be carried out. pEGFP-1 (Clontech make) is marketed as same vector for reporter assays.

[0098]

(2) Installation of a reporter gene

According to a well-known approach, the recombination vector which

enabled the manifestation of a reporter gene manifestation plasmid and the ADIPUSHIN gene of this invention in the mammals cell is produced, and simultaneous transfection of these is carried out to a cell. As a vector, although pCR3.1 (product made from in vitro JIEN), pCMV-Script (Stratagene make), etc. can be used suitably, it is not limited to these.

[0099]

As an approach of introducing a manifestation plasmid into a cell The DEAE-dextran method (Luthman, H.and Magnusson, G.(1983) Nucleic Acids Res.11, 1295-1308), A calcium phosphate-DNA coprecipitation Mr. method (Graham, F.L.and van der Eb, A.J.(1973) Virology 52, 456-457), The electric pulse terebration (Neumann, E.et al.(1982) EMBO J.1, 841-845), The RIPOFE cushion method () [ Lopata et ] Although al.(1984) Nucl.Acids Res.12, 5707-5717, Sussman and Milman (1984) Mol.Cell.Biol.4, 1641-1643, etc. can be mentioned It is not limited to these but the approach of the arbitration used widely in the technical field to which this invention belongs can be adopted. However, when a cell is the so-called floating cell, it is desirable to use approaches other than a calcium phosphate-DNA coprecipitation Mr. method. Also in which approach, it is required to use the optimum-ized transfection conditions according to the cell to be used.

[0100]

### (3) Assessment

If the cell which carried out simultaneous transfection of the ADIPUSHIN gene expression vector and reporter expression vector of this invention is cultivated in this way, the imprint of a reporter gene will be promoted by this ADIPUSHIN gene expression dependence target. Therefore, if the amount change of manifestations of the reporter gene in the case where it does not add with the case where the specimen material of arbitration is added in a culture medium under the condition which can discover a reporter gene is seen, the amount of ADIPUSHIN gene expression can be evaluated. Here, the cell transfected by the reporter expression vector survives and "the conditions which can discover a reporter gene" should just be conditions which can produce the product (reporter protein) of a reporter gene. It is the culture medium (blood serum components, such as fetal calf serum, may be added) which suited the cell strain used preferably, and culture is carried out for two - three days (most suitably for two days) at 36-38 degrees C (most suitably 37 degrees C) under the air existence containing 4 - 6% (most suitably 5%) of carbon dioxide gas.

[0101]

### (4) In addition to this (establishment of a transformed cell stock)

The test method using the cell which are a reporter gene and the ADIPUSHIN gene expression vector of this invention, and carried out the transformation of the host cell to the duplex apart from the test method

using the above passing away transgenics methods is also adoptable. In this case, it is necessary to establish a cell strain by which the manifestation of this reporter gene is promoted under the conditions which guide the ADIPUSHIN gene expression of this invention using expression vectors, such as pIND (product made from in vitro JIEN), and pTet-On (Clontech make). In creation of this transformed cell, even if the gene introduced is included in the chromosome of a host cell and piles up the passage of a host cell, being held stably is desirable. In order to choose the cell by which the transformation was carried out such, it is desirable to carry out simultaneous transfection of this selective marker that connected and carried out transfection of the selective markers (for example, neomycin (or G418) resistance gene neo etc.), such as antibiotic resistance, to the introductory gene, or was prepared separately, and the introductory gene. The cell by which the transformation was carried out stably can be chosen by using the property of this selective marker after that.

[0102]

In this way, in the obtained cell strain, the imprint of a reporter gene is promoted by this ADIPUSHIN gene expression dependence target also under the condition to which the ADIPUSHIN gene expression of this invention is guided. Therefore, if the amount change of manifestations of the reporter gene in the case where it does not add with the case where the specimen material of arbitration is added in a culture medium under the condition which can discover a reporter gene is seen, the amount of ADIPUSHIN gene expression can be evaluated.

[0103]

Process 3: Assessment of an insulin resistance improvement effect

At the last, the insulin resistance improvement effect of this examined substance is evaluated based on a difference of ADIPUSHIN or the amount of ADIPUSHIN gene expression in administration or un-prescribing a medicine for the patient. [ of an examined substance ]

That is, when ADIPUSHIN or the amount of ADIPUSHIN gene expression is increasing from under the condition of not prescribing a medicine for the patient, intentionally under the administration conditions of an examined substance, it can be estimated that this examined substance has an insulin resistance improvement effect. Here, it means that statistical significance ( $p < 0.05$ ) is in ADIPUSHIN or the amount of ADIPUSHIN gene expression under administration of an examined substance, and the conditions of not prescribing a medicine for the patient, saying "it is increasing intentionally."

[0104]

3. Kit for Assessment of Insulin Resistance Improvement Effect

This invention offers the kit for assessment of the insulin resistance improvement effect which made the index the ADIPUSHIN gene of this

invention, or the amount of manifestations of ADIPUSHIN again.

[0105]

Said kit contains at least one or more [ which is chosen from the group which consists of the following a-e ].

- a) The oligonucleotide primer which 15 – 30 base length for amplifying specifically an ADIPUSHIN gene (the array number 1 or array number 12) followed
- b) The polynucleotide probe with which the 20 – 1500 base length for combining with an ADIPUSHIN gene specifically and detecting this gene continued
- c) The solid phase-ized sample to which the polynucleotide probe given [ above-mentioned ] in b was fixed
- d) The antibody for combining with ADIPUSHIN (the array number 2 or array number 13) specifically, and detecting this ADIPUSHIN
- e) The second antibody which can be specifically combined with an antibody given [ above-mentioned ] in d

[0106]

Based on the base sequence (array numbers 1 or 12) of the ADIPUSHIN gene of this invention, according to a conventional method, it can design using commercial primer design software etc. easily, and a primer given [ said ] in a can amplify it. As an example of such a primer, the oligonucleotide which has the base sequence of a publication can be mentioned, for example to the array number 4, the array number 5, the array number 10, and the array number 11.

[0107]

Moreover, a probe given [ said ] in b is a polynucleotide specifically hybridized in the ADIPUSHIN gene of this invention, and its thing of 20 – 1500 base length extent is desirable. If it is the Northern hybridization method, specifically, the single-strand oligonucleotide or the double stranded DNA of 20 base length extent will be used suitably. Moreover, if it is a microarray, the double stranded DNA of 100 – 1500 base length extent or the single-strand oligonucleotide of 20 – 100 base length extent will be used suitably. On the other hand, the Gene Chip system of Affimetrix has good single-strand oligo of 25 base length extent. As for especially these, it is desirable to design as a probe which the array singularity which exists in 3' untranslation region of the ADIPUSHIN gene of this invention hybridizes specifically into a high part. The label of for example, enzyme labeling, a radioactive indicator, fluorescent labeling, etc. may be carried out with the suitable indicator, and these primers and probes may be embellished by the biotin, the phosphoric acid, the amine, etc. As an example of such a probe, the oligonucleotide which has the base sequence of a publication can be mentioned to the array number 6.

[0108]

Moreover, a solid phase-ized sample given [ said ] in c is produced by fixing a probe given [ said ] in b to the solid phase of a glass plate, a nylon membrane, a micro bead, a silicon chip, etc. Although 2.1 already explained such a solid phase-ized sample and its production approach, a gene chip, a cDNA array, an oligo array, a membrane filter, etc. can be mentioned, for example.

[0109]

An antibody said d and given in e is producible by the approach indicated to 2.2. The label of for example, enzyme labeling, a radioactive indicator, fluorescent labeling, etc. may be carried out with the suitable indicator, and this antibody may be suitably embellished by the biotin etc.

[0110]

The kit of this invention may contain suitably other reagents required for the assessment approach of the insulin resistance improvement effect of the examined substance concerning this inventions, such as detection of hybridization, the indicator of a probe, and a label object, etc. if needed besides the above-mentioned component.

[0111]

#### 4. Insulin Resistance Improvement Agent

In the type 2 diabetes model animal, when this invention persons did the superfluous manifestation of the ADIPUSHIN gene, they checked that the indication of an insulin resistance improvement was seen. In this result, ADIPUSHIN and an ADIPUSHIN gene show a useful thing to the therapy of insulin resistance.

That is, this invention offers the insulin resistance improvement agent containing Homo sapiens ADIPUSHIN or a Homo sapiens ADIPUSHIN gene.

[0112]

##### 4.1 Insulin Resistance Improvement Agent Containing Homo Sapiens ADIPUSHIN Gene

Insulin resistance can be made to improve by medicating with a recombination vector (gene transfer vector) including all the open reading frame arrays of a Homo sapiens ADIPUSHIN gene the patient who has insulin resistance, such as type 2 diabetes, and making him discovered in this patient's cell.

[0113]

Here, if the transgenics to a human cell is possible for the vector promotor system used, it is good. For example, a MoMLV vector, a Herpes virus vector, an adenovirus vector, an AAV vector, a HIV vector, S1V vector, and the virus vector of the Sendai Virus vector first class are mentioned. The macromolecule carrier which uses the complex of Lynn \*\* calcium and a nucleic acid, ribosome, cation lipid complex, Sendai Virus liposome, and the

poly cation as a principal chain as vectors other than a virus is usable. Furthermore, the approach of electroporation, a gene gun, etc. may be used. [0114]

A promotor will not be especially limited, if a gene can be made to discover within a human cell. For example, adenovirus, a cytomegalovirus, a human immunodeficiency virus, The simian virus 40, the Rous sarcoma virus, a herpes simplex virus, A murine leukemia virus, a SHIMBISU virus, a hepatitis A virus, a hepatitis B virus, A hepatitis C virus, papillomavirus, a human T cell leukemia virus, The promotor of the virus origins, such as an influenza virus, a Japanese encephalitis virus, a JC virus, a parvovirus B19, and a poliovirus, The promotor to whom a manifestation is guided with chimera mold promoters, such as promoters of the mammals origin, such as albumin, SRalpha, a heat shock protein, and an elongation factor, and a CAG promotor, a tetracycline, a steroid, etc. is mentioned. In addition, the virus vector of shoe DOTAIPU may be used. The shoe DOTAIPU virus vector which permuted the Env protein which is coat protein of HIV as the example by the VSV-G protein which is coat protein of a small varicella nature stomatitis virus (Vesicular stomatitis Virus:VSV) is mentioned (Naldini L etc.: Science 272 263 (1996)).

[0115]

#### 4.2 Insulin Resistance Improvement Agent Containing Homo Sapiens ADIPUSHIN

The patient who has insulin resistance, such as type 2 diabetes, can be made to improve insulin resistance by prescribing the constituent containing Homo sapiens ADIPUSHIN or Homo sapiens ADIPUSHIN for the patient.

[0116]

First, the ADIPUSHIN protein which connects all the open reading frame arrays of an ADIPUSHIN gene with a suitable vector promotor system, introduces them into host cell lineage, and targets them is made produce and refined [ extract and ], and is rearranged, and ADIPUSHIN is produced.

[0117]

what was enumerated by the vector promotor system of the preceding clause as said vector — the vector in which transgenics is possible is mentioned to the mammals, especially a human cell inside. what similarly was enumerated by the vector promotor system of the preceding clause as an usable promoter — the promoter in whom gene expression is possible is mentioned by the mammals, especially the human cell inside. Moreover, the promotor who can be discovered within Escherichia coli hosts, such as a Lac promotor, can also use it.

[0118]

As said host cell lineage, an animal cell, an insect cell, a plant cell, Escherichia coli, a growth hen's egg, etc. are mentioned, for example.

Furthermore, the approach of repeating the approach of dissolving a cell membrane using the surfactants and enzymes which carry out ize [ of the cell / HOMOJU ], such as an approach and SDS, for example as an approach of extracting the protein produced from the host cell lineage which carried out transgenics, sonication, freezing, and fusion etc. is mentioned. The recombination ADIPUSHIN protein obtained by which approach is also refined with a conventional method. For example, general biochemical models using column separation, polyacrylamide gel, etc. using the centrifugal separation and the ion exchange column using ultracentrifuge or density gradient centrifugation, an affinity column (for example, the above-mentioned specific antibody is used), an opposite phase column, etc., such as gel separation, can use for purification.

[0119]

It is mixed with the support or the diluent permitted pharmacologically according to a well-known approach, and ADIPUSHIN which was manufactured as mentioned above and refined can be pharmacologically pharmaceutical-preparation-ized to a useful constituent. Said pharmacological constituent may contain said polypeptide of 1 or two or more effective amounts in therapy. Suitable support and a diluent are indicated by Remington's Pharmaceutical Sciences etc., for example.

[0120]

Although especially definition is not carried out, as for the pharmaceutical form which was suitable for administration about said protein, it is desirable to be prepared as injections like the pharmacological constituent containing much Homo sapiens protein already used as a remedy. Speaking more concretely, letting ADIPUSHIN be injections by dissolving in suitable solvents, such as water, a physiological saline, and the buffer solution that carried out tonicity adjustment. In that case, it may add as a protective agent and a polyethylene glycol, a glucose, various amino acid, a collagen, albumin, etc. may be prepared. Moreover, it is also possible to carry out embedding of the polypeptide to inclusion bodies, such as ribosome, and to medicate them with it.

[0121]

6 In Addition to this

6.1 Prediction of Insulin Resistance (Susceptibility)

ADIPUSHIN concerning this invention and its amount of gene expression have high possibility of reflecting the insulin resistance (susceptibility) of the animal. This test subject's insulin resistance can be predicted by following, for example, measuring the amount of ADIPUSHIN gene expression in a test subject's blood and cell. Carrying out to accuracy more can carry out possible [ of such prediction ] by carrying out comparison analysis of the gene expression profiles, such as the factor which reflects other insulin



resistance (susceptibility) with an ADIPUSHIN gene, for example, TNF- $\alpha$ , ADIPONE cutin, etc., on the whole.

## 6.2 Production of Model Animal Which Has Insulin Resistance

By reducing artificially ADIPUSHIN and ADIPUSHIN gene expression concerning this invention, it is also possible to produce the animals (for example, mouse etc.) which have insulin resistance. For example, the ribozyme and RNAi to an ADIPUSHIN gene are introduced into an animal, and if an expressive change similar to the insulin resistance venereal disease voice represented by Homo sapiens type 2 diabetes appears, research of insulin resistance venereal disease voice or its improvement agent can be done using this animal. Similarly, the model animal which has insulin resistance is producible by introducing an anti-ADIPUSHIN antibody into a direct animal.

[Example]

[0122]

Hereafter, although an example explains this invention to a detail further, this invention is not limited to these examples.

### Example 1: GeneChip analysis

Chip analysis was performed by the approach of indicating below according to the manifestation analysis engineering manual (Expression Analysis Technical Manual) of an AFI metrics company.

#### a) Preparation of a mouse

As a type 2 diabetes model mouse, the KK/Ta mouse (made in Japanese Clare, N= 2) was prepared. Moreover, the salts permitted as an insulin resistance improvement agent on

5-[4-(6-methoxy-1-methylbenzimidazol-2-ylmethoxy) benzyl] thiazolidine -2 indicated by the EP disclosure No. 745600 official report, 4-dione, and its pharmacology (hydrochloride etc.) (here) the hydrochloride of 5-[4-(6-methoxy-1-methylbenzimidazol-2-ylmethoxy) benzyl] thiazolidine -2 and 4-dione is hereafter called "compound A." It prepared. each mouse -- compound A -- a weight ratio -- 0.0003% -- it is -- one day and three days -- or mixed feed administration was carried out for eight days. As control, the mouse (N= 2) was similarly bred with the feed which does not contain compound A. All the mice extracted liver after administration termination, in order to prepare all RNA.

[0123]

#### b) Preparation of all RNA

The extracted liver extracted all RNA according to the attached protocol using all the reagents for an RNA extract (TRIzol reagent: product made from Gibco BIARUERU). Subsequently, after dissolving all obtained RNA in the TRIzol reagent again and performing a phenol and a chloroform extraction, ethanol precipitate was performed, it dissolved in the pure water which does

not contain an ribonuclease (henceforth "RNase"), and the obtained pellet was used as all RNA solutions.

[0124]

c) Composition of cDNA

According to the above-mentioned manual publication, composition and purification of cDNA were performed by using all RNA of obtained 10microeach g as a start ingredient. It is the following array which specifically includes reverse transcriptase (SuperscriptII, product made from Gibco BIARUERU), and T7 promotor array after keeping it warm for 10 minutes, quenching all RNA and denaturing it at 65 degrees C. :

5'-ggccagtgaatgtaatacagactcactatagggaggcggtttttttttttttttttttttttt-3'  
(array number 3)

The single strand cDNA was compounded using the oligo (dT) primer (OligoExpress, Amersham Pharmacia manufacture) which \*\*\*\*. The composite reaction condition could be 42 degrees C and 1 hour. Then, DNA polymerase I (product made from Gibco BIARUERU) was added into this system of reaction, and cDNA was used as the double strand. The reaction condition could be 16 degrees C and 2 hours.

d) Composition of cRNA

According to the publication of the above-mentioned manual, cRNA was produced by using as mold the cDNA whole quantity obtained as mentioned above. (The reaction condition could be 37 degrees C and 4.5 hours) . cRNA of 20microg obtained by this actuation was fragmented, and an equivalent for 15microg was added to the probe solution inside.

[0125]

e) Production of a probe solution

The transformation Escherichia coli holding the plasmid DNA for control cRNA production added to a probe solution was purchased from the American type culture collection (ATCC) (pglks-bioB (ATCC87487), pglks-bioC (ATCC87488), pglks-bioD (ATCC87489), and pglks-cre (ATCC87490)). After cultivating these transformation bacilli in TB culture medium (12g [ per l. ] bacto trypton, a 24g bacto yeast extract, K<sub>2</sub>HPO<sub>4</sub> of 2.3g of KH<sub>2</sub>PO<sub>4</sub>(s) 4 and 12.5g, and 4ml glycerol are included) which contains 200ml ampicillin [ 100microg //ml ], respectively, they collected plasmids with the alkaline process. These plasmids were further refined by cesium chloride density gradient ultracentrifugation. Hereafter, both the production approach of Control cRNA and the presentation of a probe solution followed the above-mentioned manual publication.

[0126]

f) Hybridization

As the probe obtained as mentioned above and a chip made to hybridize, mouse MG-U74 (U74A, U74B, U74C) by the AFI metrics company was used.

Washing actuation of hybridization and after that was performed according to the above-mentioned manual publication. In addition, hybridization conditions were made into 45 degrees C and 18 to 22 hours.

g) Analysis

Data analysis of the chip which performed hybridization actuation was performed according to the above-mentioned manual publication.

[0127]

The "Fold Change value" showing the amount of relative manifestations of each gene showed gene expression level. A Fold Change value expresses the amount of relative manifestations of each gene in the medication group to drugs the group non-prescribing a medicine for the patient (control) with this example. For example, when the Fold Change value of a certain gene is 10, many the gene is discovered 10 times from the group non-prescribing a medicine for the patient in a compound A administration group.

[0128]

In this way, as a result of analyzing the Fold Change value of each gene, the gene of identification number (probe set No.) 99671\_at was identified as what is carrying out the high manifestation from the group non-prescribing a medicine for the patient in the liver of an administration group. The array information of an AFI metrics company showed that it was what makes this array the nucleotide sequence which carries out the code of mouse ADIPUSHIN, and is registered into the GenBank database (GenBank Accession No.:NM\_013459).

[0129]

The Fold Change value of identification number:99671\_at (mouse ADIPUSHIN) in a KK/Ta mouse administration group was shown in a table 1 (data of a U74A chip). From this result, as for the ADIPUSHIN gene, it was checked by the 1st day of the administration by the administration group to the group non-prescribing a medicine for the patient in 3.1 times and the 3rd day 19 being times and that it is discovered 18.9 times more highly [ in the 8th day ].

[0130]

[A table 1]

## probe set No. 99671\_at の Fold Change 値

	1 日目	3 日目	8 日目
化合物 A 非投与群	4. 5	5. 4	5. 9
化合物 A 投与群	19. 5	152	157
発現量比 (投与群/投与群)	3. 1	19	18. 9

[0131]

Example 2: Measurement of the amount of ADIPUSHIN manifestations

a) Preparation of cDNA

According to the example 1, liver was extracted from the mouse (control) which does not prescribe a mixed feed \*\*\*\*\* KK/Ta mouse and compound A for the patient for compound A for one day, three days, or seven days by the dosage of 0.0003% of heavy quantitative ratios, and all RNA solutions were prepared.

[0132]

Pure water was added to all obtained RNA 6 microg, the whole quantity was set to 40 microl, it cooled to 4 degrees C rapidly, and 65 degrees C of thermal denaturation were performed, after keeping it warm for 10 minutes. All these RNA solutions are poured distributively in two 20 micro every 1 tubes [ micro ] for thermal SAIKURA. respectively -- alike -- a cDNA composition kit (a first strand cDNA composition kit --) Dithiothreitol solution 1 microl of the Amersham Pharmacia manufacture, NotI-oligo-(dT)-primer 1 microl diluted with the pure water which does not contain RNase 5 times, Bulk first strand mix 11 microl was added, it was kept warm at 37 degrees C for 1 hour using thermal SAIKURA (DNA engine PTC-200, MJ research company make), and the single strand cDNA was compounded.

[0133]

The pure water which does not contain RNase of 17 microl was added to one tube after reaction termination, and it considered as the single strand cDNA solution for calibration-curve creation. The pure water which does not contain RNase of 217 microl was added to another tube, and it considered as the single strand cDNA solution for quanta. By using a nucleic-acid purification spin column (the chroma-spin 100, Clontech make) according to an attached protocol, these single strand cDNA solution was refined and it considered as the mold single strand cDNA solution for TaqMan PCR.

[0134]

## b)TaqMan PCR

On the other hand, the oligonucleotide which has the following array as a primer for mouse ADIPUSHIN DNA magnification for TaqMan PCR was compounded (OligoExpress, Pharmacia Corp., Amersham).

5'-gagtgtcaat catgaaccgg a-3' (array number 4)

5'-tggttaatggt gactaccccg tc-3' (array number 5)

[0135]

Moreover, it has the following array as a probe for mouse ADIPUSHIN DNA detection for TaqMan PCR, and the oligonucleotide which combined reporter coloring matter Fam with the five prime end, and combined Tamara of a reporter quenching object with the three-dash terminal was compounded (TaqMan full me a cent probe, Applied Biosystems Japan make).

5'-aacctgcaat ctgcgcacgt acca-3' (array number 6)

[0136]

On the other hand, in order to standardize the amount of manifestations of mouse ADIPUSHIN, we decided to measure simultaneously the amount of 36 B4 mRNA manifestations which is ribosomal protein, and the oligonucleotide which has the following array as a primer for mouse 36 B4 magnification for TaqMan PCR was compounded (oligoExpress, Amersham Pharmacia manufacture).

5'-gctccaagca gatgcagca-3' (array number 7)

5'-ccggatgtga ggcagcag-3' (array number 8)

[0137]

Moreover, it has the following array as a probe for mouse 36 B4 detection for TaqMan PCR, and the oligonucleotide which combined reporter coloring matter Fam with the five prime end, and combined Tamara of a reporter quenching object with the three-dash terminal was compounded (TaqMan full me a cent probe, Applied Biosystems Japan make).

5'-caagaacacc atgatgcgca aggc-3' (array number 9)

[0138]

## c) The quantum of the amount of ADIPUSHIN manifestations

The sample prepared as mentioned above is used and it is TaqMan. The quantum of the amount of manifestations of mouse ADIPUSHIN by the PCR method was performed.

As for the presentation of an PCR reaction solution, the square opposite side and hard flow side set per one sample, mouse liver origin mold single strand cDNA solution 5microl, and a primer (100pmol/mul) to 0.1microl (last concentration 200nM), probe (6.5microM) 1.5microl (last concentration 195nM), mixed liquor (TaqMan universal PCR master mix, Applied Biosystems Japan make) 25microfor PCR magnification I, and ultrapure water 18.3microl. In addition, the single strand cDNA solution for calibration-curve creation set the relative value of undiluted solution concentration to "625" for

convenience, repeated dilution 5 times after that, created five steps of dilution sequences, a concentration value "625", "125", "25", "5", and "1", and prepared the reaction mixture which made the presentation of those other than a single strand cDNA the same as the above.

[0139]

These reaction mixture was put into the well of 96 hole reaction plate (micro amplifier optical and 96 well reaction plate, Applied Biosystems Japan make), and PCR was performed using thermal SAIKURA only for TaqMan PCR, and a detector (ABI7700, Applied Biosystems Japan make). A reaction is 50 degrees C and measured [ 95 degrees C ] the amount of luminescence of reporter coloring matter for the reaction for 1 minute for every [ 40 cycle repeat and ] cycle at 60 degrees C for 15 seconds by 95 degrees C after incubation for 10 minutes for 2 minutes.

[0140]

d) Analysis

The magnification curve of the DNA fragment which carries out the code of 36 B4 and each of ADIPUSHIN from the amount of luminescence of the reporter coloring matter for every cycle was created. the calibration curve which took concentration along the axis of abscissa from the magnification curve of the dilution sequence of the single strand cDNA solution for calibration-curve creation, and took the number of cycles along the axis of ordinate -- creating -- each sample for manifestation quanta -- the logarithm -- the number of cycles beyond the fixed amount of luminescence set as arbitration at the magnification term was plotted on the calibration curve, and the relative amount of manifestations was computed. The amount of manifestations of ADIPUSHIN amended with the value of the amount of manifestations of 36 B4 in the same sample.

The amount of relative manifestations of the ADIPUSHIN gene in each mouse was shown in drawing 1 . this result to an ADIPUSHIN gene -- an insulin resistance improvement agent -- it was checked that a manifestation increases remarkably by administration of compound A.

[0141]

Example 3: Production of recombination adenovirus

a) Preparation of ADIPUSHIN cDNA

In order to build the manifestation system using an adenovirus vector, cDNA which carries out the code of the amino acid of mouse ADIPUSHIN was prepared using the PCR method.

[0142]

Liver was specifically extracted for the powder food in which compound A was made to mix 0.0003% by the weight ratio like an example 1 from the \*\*\*\*\* KK/Ta mouse for one week, and all RNA solutions were prepared. All obtained RNA solutions Pure water was added to 6microg, the whole

quantity was set to 40microl, it cooled to 4 degrees C rapidly, and 65 degrees C of thermal denaturation were performed, after keeping it warm for 10 minutes. All these RNA solutions are poured distributively in two 20micro every 1 tubes [ micro ] for thermal SAIKURA. respectively -- alike -- a single strand complementary DNA (henceforth "cDNA") composition kit (a first strand cDNA composition kit --) Dithiothreitol solution 1microl of the Amersham Pharmacia manufacture, NotI-oligo-(dT)-primer 1microl diluted with the pure water which does not contain RNase 5 times, Bulk first strand mix 11microl was added, it was kept warm at 37 degrees C for 1 hour using thermal SAIKURA (DNA engine PTC-200, MJ research company make), and the single strand cDNA was compounded.

[0143]

The pure water which does not contain RNase of 217microl in a tube was added after reaction termination, and it considered as the single strand cDNA solution. By using a nucleic-acid purification spin column (the chroma-spin 100, Clontech make) according to an attached protocol, these single strand cDNA solution was refined and it considered as the mold single strand cDNA solution for PCR.

[0144]

Moreover, the oligo DNA which has the following array as a primer for PCR magnification is compounded (Pharmacia Corp., Amersham), and it is ADIPUSHIN. It considered as the primer for cDNA cloning.

5'-agg gaa ttc atg cac agc tcc gtg tac ttc gtg-3' (array number 10)

5'-agg gga tcc tca gga tgt cat gtt acc att tgt-3' (array number 11)

[0145]

platinum Pfx(product made from Gibco BIARUERU)0.5microl was mixed to the exclusive buffer solution (product made from Gibco BIARUERU) as ten primer pmol(s) each for above-mentioned mold single strand cDNA1microl and magnification, 10mM dNTPs solution (TAKARA SHUZO CO., LTD. make) 3microl, and heat-resistant DNA polymerase, and it was referred to as total 50microl. A magnification reaction is 20 cycle \*\*\*\*\*, using for 30 seconds at 54 degrees C for 30 seconds, and using [ using thermal SAIKURA (DNA engine PTC-200, MJ research company make), by 94 degrees C make for 30 seconds at X degrees C for 30 seconds, and it makes for 2 minutes 1 cycle at 72 degrees C, and / change X with 74->70->66->62->58, ] for 2 minutes as 1 cycle at 72 degrees C by 94 degrees C, three cycles each and after that.

[0146]

It developed by carrying out electrophoresis in 1% agarose gel, and the magnification product collected DNA near [ which is expected as an object product ] about 800 bp using REKOCHIPPU (TAKARA SHUZO CO., LTD. make), performed ethanol precipitate and refined it. the obtained DNA

precipitate -- K buffer solution (TAKARA SHUZO CO., LTD. make) -- dissolving -- EcoRI and BamHI -- every [ two Unit each ] -- it added, and 37 degrees C was processed for 2 hours, and electrophoresis was again performed by agarose 1%, it developed, the target DNA bands were collected, and it dissolved in the ultrapure water of 10microl. After digesting by this DNA solution 4microl, EcoRI, and BamHI, pSG5 vector (Stratagene make) 100ng processed with bacteria origin ARUKARU phosphatase was mixed, and after 5microl Adding and mixing 20microl and B liquid and making A liquid of the DNA ligation kit version I (TAKARA SHUZO CO., LTD. make) react at 16 degrees C for 1 hour, it refined by performing ethanol precipitate. It cultivated 37 degrees C and overnight on the agar medium which dissolves DNA which precipitated in ultrapure water, introduces a plasmid into Escherichia coli by the electroporation method in addition to a JM109 electro cel (TAKARA SHUZO CO., LTD. make), and contains ampicillin by 100microg [ /ml ] concentration. After cultivating the obtained transformant in LB culture medium, the plasmid was extracted, and the array of introduced cDNA was determined by the die termination method.

[0147]

b) Preparation of a recombination adenovirus

The recombination adenovirus for carrying out a forcible manifestation produced obtained ADIPUSHIN cDNA using the commercial kit (an adenovirus Expression vector kit, TAKARA SHUZO CO., LTD. make). Namely, it used for the following actuation by using what digested the plasmid DNA obtained for the preceding clause with restriction enzymes EcoRI and BamHI, and graduated the end of the DNA fragment of about 800 obtained bp(s) as an insertion DNA fragment.

[0148]

Moreover, cosmid pAxCA-adipsin which inserted the insertion DNA fragment in the restriction enzyme SwaI recognition site of the cosmid vector pAxCAwt (it attaches to an adenovirus Expression vector kit) currently designed so that a cytomegalovirus enhancer and a fowl beta-actin promotor may be discovered was produced. Using the calcium phosphate transfection system (Amersham Pharmacia manufacture), KO transfection of pAxCA-adipsin DNA or pAxCADNA for contrast, and the end protein joint viral DNA (it attaches to DNA-TPC and an adenovirus Expression vector kit) was carried out to 293 cells (ATCC CRL1573), they were rearranged, adenovirus was isolated, and the pan was made to amplify in 293 cells. The recovery from 293 cells of the virus made to amplify performed sonication (made in [ B-1200 ] Branson is used) of 30 second x4 time into virus infection 293 cell first, and was performed by subsequently repeating purification by cesium chloride density-gradient centrifugation twice. In this way, the recombination adenovirus (henceforth "Ad/Adipsin") containing



ADIPUSHIN DNA and the recombination adenovirus (henceforth "Ad/empty") which does not contain ADIPUSHIN DNA for contrast were prepared.

Cryopreservation of it was carried out below  $-70$  degrees C after dialyzing the obtained virus liquid twice at  $4$  degrees C to PBS which added glycerol 10% until it used it.

[0149]

Example 4: The manifestation by in vivo of ADIPUSHIN

a) Inoculation of a recombination adenovirus

Ad/Adipsin or Ad/empty produced in the example 3 was diluted with the physiological saline so that it might be set to  $1 \times 10^{10}$  pfu/ml,  $2 \times 10^9$  pfu/ml, and  $5 \times 10^8$  pfu/ml, and caudal vein injection inoculated it into three 20-weeks old male KK/Ta mice every [ 200micro / l ], respectively.

[0150]

b) Measurement of the parameter in blood

3, 8, and 14 days after inoculation, pass from the caudal vein of each mouse and it collects blood using the Palin processing hematocrit tube. With a desk centrifuge, by 5200rpm, carry out centrifugal twice and plasma is separated for 15 minutes. The kit for glucose measurement (glucose CII-Test Wako, Wako Pure Chem make) is used. The blood sugar level The insulin value in blood was measured for neutral fat concentration using the insulin measurement kit (made in the Morinaga biochemistry lab) using the kit for neutral fat measurement (triglyceride E-Test Wako, the Wako Pharmaceuticals company make), respectively. Change of various parameters is shown in drawing 2 (A-E).

Consequently, by the Ad/empty inoculation mouse group, the lowering inclination was accepted in the blood sugar level, neutral fat concentration, and the insulin value in blood by the Ad/Adipsin inoculation mouse group to a significant difference not having been accepted in the parameter in blood.

[0151]

c) Western blotting

Moreover, the plasma of an adenovirus infection mouse group with which the difference was accepted in the parameter in these blood was developed by SDS-PAGE under reduction conditions using 10-20% polyacrylamide density gradient gel (the multi-gels 4/20, the first chemicals company make).

The band was imprinted from polyacrylamide gel to the nitrocellulose membrane (Bio-Rad make) on condition that 15V after electrophoresis for 45 minutes using gel membrane imprint equipment (TRANS-BLOT SD, Bio-Rad make) in the imprint buffer solution (a 192mM glycine, 20% methanol, 2Vmm tris). About the nitrocellulose membrane after an imprint, western blot analysis which used the anti-mouse ADIPUSHIN antibody (p-16, product made from St. KURUZU) was performed. That is, after washing a

nitrocellulose membrane by PBS (henceforth "PBST") containing 0.1% of Tween20 first (it is subsequently 2 times about for 5 minutes once in for 15 minutes at a room temperature), it put into the plastics bag (high buri back, Cosmobio make), 20ml of 0.1% PBST(s) which contain BSA (pierced earring company make) 1% was added, and it shook at 4 degrees C overnight. an after that membrane -- the inside of ejection and 0.1%PBST -- x during 15 minutes -- subsequently it washed twice [ during 5 minutes / x ] once. The membrane was moved to a new plastics bag after washing, an anti-ADIPUSHIN antibody (100 time dilution) and 5ml of 0.1%PBST(s) which contain BSA 1% were added, and it shook at the room temperature for 1 hour. 1 hour after and a membrane -- 0.1% PBST of ejection -- x during 15 minutes -- it washed twice [ during 5 minutes / x ] once. Then, the membrane was moved to a new plastics bag, 5ml of solutions which diluted the horseradish peroxidase indicator anti-goat IgG antibody (Bio-Rad make) with PBST 1000 times 0.1% was put in, and it shook at the room temperature for 1 hour. 1 more hour after and a membrane -- ejection and 0.1%PBST -- x during 15 minutes -- subsequently it washed twice [ during 5 minutes / x ] once. The membrane was placed on the wrap film after washing, and mouse ADIPUSHIN was detected using the ECL Western-blotting detection solution (Amersham Pharmacia manufacture) (after placing a membrane on a wrap film and dipping in an ECL Western-blotting detection solution for 1 minute, the X-ray film was exposed (for 3 seconds)). Consequently, the band specific in the plasma of the KK/Ta mouse with which Ad/Adipsin was infected was detected ( drawing 3 ).

[0152]

When the superfluous manifestation of the ADIPUSHIN gene was carried out by that ADIPUSHIN gene expression is guided notably and the liver of the mouse concerned from the above result with the diabetes-mellitus model mouse which prescribed the insulin resistance improvement agent for the patient, it was checked that the indication of an insulin resistance improvement appears. That is, ADIPUSHIN and an ADIPUSHIN gene can serve as insulin resistance and an index of the improvement. Therefore, if ADIPUSHIN and the amount of ADIPUSHIN gene expression are investigated, the insulin resistance improvement effect of the test compound concerned can be evaluated. [ compound A ] [ in / a mouse is medicated with a suitable test compound instead of, and / by the same procedure as the above / this mouse ]

[0153]

The example of reference: Manufacture of

5-[4-(6-methoxy-1-methylbenzimidazol-2-ylmethoxy) benzyl] thiazolidine -2 and 4-dione hydrochloride

The results object which was compounded [ Europe patent application

disclosure / No. 745600 ] by the approach of a publication and which added ethyl acetate after condensing mixture (5-[4-(6-methoxy-1-methylbenzimidazol-2-ylmethoxy) benzyl] thiazolidine-2 and 4-dione 10.6g and 4 convention hydrochloric-acid-1,4-dioxane 100ml), and deposited was separated, ethyl acetate washed, and 11.0g of the object compounds which have the 275 to 277 degree C melting point was obtained.

[0154]

<sup>1</sup>H-nuclear-magnetic-resonance spectrum: <sup>1</sup>H-nuclear-magnetic-resonance spectrum (400MHz):delta (ppm) measured to the internal standard using TMS (tetramethylsilane) is as follows among delta(ppm):pile dimethyl sulfoxide.

[0155]

3.11 (1H, Dd, J= 14Hz and 9Hz, and 3.34 (1H, Dd, J= 14Hz, and 4Hz), 3.89 (3H, s), 3.98 (3H, s), and 4.91 (1H, dd, J= 9Hz, and 4Hz), 5.64 (2H, s) and 7.14 (2H, d, J= 9Hz), 7.15 7.25 (1H, d, J= 9Hz) (2H, d, J= 9Hz), 7.50 (1H, s), 7.70 (1H, d, nine Hz), and 12.04 (it disappears by 1H, s, and D2O addition).

[Array table free text]

[0156]

Explanation of an array number 3-artificial array: Synthetic DNA (primer)

Explanation of an array number 4-artificial array: Synthetic DNA (primer)

Explanation of an array number 5-artificial array: Synthetic DNA (primer)

Explanation of an array number 6-artificial array: Synthetic DNA (probe)

Explanation of an array number 7-artificial array: Synthetic DNA (primer)

Explanation of an array number 8-artificial array: Synthetic DNA (primer)

Explanation of an array number 9-artificial array: Synthetic DNA (probe)

Explanation of an array number 10-artificial array: Synthetic DNA (primer)

Explanation of an array number 11-artificial array: Synthetic DNA (primer)

[Brief Description of the Drawings]

[0157]

[Drawing 1] Drawing 1 shows the amount of relative manifestations of the ADIPUSHIN gene by TaqMan PCR.

[Drawing 2] Drawing 2 shows change of the various parameters by installation of the ADIPUSHIN gene in a type 2 diabetes model mouse. (A: Blood glucose, the insulin in B: blood, C: weight, the triglyceride in D: blood, the amount of E: baiting)

[Drawing 3] Drawing 3 shows the result of Western blot.

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[Translation done.]

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- 1.This document has been translated by computer. So the translation may not reflect the original precisely.
- 2.\*\*\*\* shows the word which can not be translated.
- 3.In the drawings, any words are not translated.

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**DESCRIPTION OF DRAWINGS**

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